



PATENTS

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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JUL 16 2002

In re Application of: )

O'REILLY ET AL. )

Examiner: Huff, S. )

TECH CENTER 1600/2900

Serial No. 09/154,302 )

Art Unit: 1642 )

Filed: SEPTEMBER 16, 1998 )

For: METHODS OF INHIBITING )  
ANGIOGENESIS WITH )  
ENDOSTATIN PROTEIN )COPY OF PAPERS  
ORIGINALLY FILEDDECLARATION OF BJORN R. OLSEN, PH.D. UNDER 37 CFR § 1.132

I, BJORN R. OLSEN PH.D., hereby declare as follows:

1. I am a full Professor in the Department of Cell Biology at Harvard Medical School and have conducted and directed research in anatomy, physiology and molecular biology. I have served as the Chairman for the Program in Cell and Developmental Biology at Harvard Medical School, Co-Chairman for the New York Academy of Sciences Conference on "Structure, Molecular Biology, and Pathology of Collagen", full Professor in the Department of Biochemistry at Rutgers Medical School, and President for the International Society for Matrix Biology. In addition, I have also chaired numerous conferences, including the Gordon Conference "Structural Macromolecules Collagen" and the New York Academy of Sciences Conference on "Molecular and Developmental Biology of Cartilage". My achievements in the areas of molecular biology and medicine have been recognized by several awards such as the MERIT Award for Scientific Excellence (1992 and 1993 awarded by the National Institutes of Health), Distinguished Faculty Award (Harvard University) and Honorary Doctorate Degrees in Science (University of Medicine and Dentistry of New Jersey and University of Oslo, Norway). I have participated on the scientific advisory boards of several major biomedical and biopharmaceutical companies such as Metra Biosystems, Inc. and OsteoArthritis Sciences, Inc. In addition, I have served on the Board of Directors of Organogenesis, Inc. since 1994. At both Rutgers Medical School and Harvard Medical

School, I have participated in numerous committees, including Faculty Council and Subcommittee of Professors, and Standing Committee on Student Prizes and Awards in the Faculty of Medicine. I have served and continue to serve on various Editorial Boards of major scientific journals such as the Journal of Cell Biology, Current Opinions in Cell Biology and Journal of Biological Chemistry. My teaching experience encompasses many areas of biology and medicine such as anatomy, physiology, histology, gross anatomy, biochemistry, cell biology, and developmental biology.

My major areas of research interests include the role of the extracellular matrix in embryonic development, skeletal cell and molecular biology, molecular genetics, molecular pathology and the structure, biosynthesis and function of collagens. I have published over 270 scientific papers in these and other related areas. A complete listing of these papers, publications, theses and/or dissertations accompanies the copy of my *curriculum vitae*, which is attached hereto as Exhibit A.

2. I have reviewed U.S. Patent Application Serial No. 09/154,302, entitled "Methods of Inhibiting Angiogenesis with Endostatin Protein," and understand the claimed invention to comprise a method of inhibiting angiogenesis in an individual comprising the administration of a protein wherein the protein is a fragment of a C-terminal non-collagenous (NC1) region of a collagen protein.

The Office Action, dated September 28, 2001, rejected applicants' claims stating in part that the specification, while being enabling for methods using endostatin from collagen XVIII, does not reasonably provide enablement for the broad claims pertaining to the use of any inhibitory protein molecule that has an NC1 region.

The Office Action further stated that applicants' specification indicates that proteins that share a common or similar N-terminal amino acid sequence with endostatin would possess antiangiogenic activity, which according to the Action indicates that sequence similarity is needed.

The Action concluded that although applicants "argued that all NC1 regions are known to have a helical structure, this structure has not been correlated to antiangiogenic activity."

Being one skilled in the relevant art, I respectfully disagree with the rejections set forth in September 28, 2001 Office Action for the reasons provided below.

3. Applicants have defined the claimed invention according to both structure and function, and, based on applicants' description together with the level of knowledge in this field at the time the application was filed, one skilled in the art would easily be able to identify antiangiogenic proteins derived from collagen proteins. The structural feature of importance is the C-terminal noncollagenous region (NC1) of collagen proteins. The functional feature of importance is antiangiogenic activity.

4. As indicated in the specification (page 37, lines 3-15) and known to those skilled in the art, the NC1 region is a defined structural feature that is present in many collagen proteins. (See, for example, the chapters on collagens in the text, *GUIDEBOOK TO THE EXTRACELLULAR MATRIX, ANCHOR, AND ADHESION PROTEINS*, second edition, Kreis and Vale, eds., Oxford University Press, 1999, pp. 380-408, which were authored by Yoshifumi Ninomiya and myself, a copy of which is attached as Exhibit B.)

The collagens comprise a large family of *genetically* distinct, but *structurally* related proteins. A prominent and common feature of most of the collagens is that they contain both collagenous and noncollagenous regions. Specifically, the collagenous regions of collagen molecules consist of the conserved structural feature of three polypeptide chains, called alpha chains, that wind around each other in a right-handed helix in each molecule to form a characteristic collagen triple helix. The ability of collagenous proteins to form structures of high tensile strength is based on the rigid structure of collagen molecules. Collagen polypeptides contain one or more blocks of Gly-Xaa-Yaa repeats, in which Yaa frequently represents a proline or hydroxyproline residue. The presence of such sequence repeats allows groups of three polypeptides to fold into triple-helical domains that are rigid and inextensible. The use of such triple-helical domains was initially thought to be limited to molecules that make up collagen fibrils in tissues, but it is now known that such domains are present in a majority of collagen proteins. As further stated by Rehn and Pihlajaniemi, most collagen molecules contain noncollagenous sequences at their termini, and several types also have them as

interruptions separating adjacent triple helical regions (*Proc. Natl. Acad. Sci. USA* 91:4234, 1994, attached as Exhibit C). Accordingly, those skilled in the art easily recognize the highly conserved features of collagens consisting of right-handed triple helixes as the collagenous regions, and intervening areas and terminal portions as the noncollagenous regions.

5. The Office Action further states that NC1 regions are ill-defined, in part because the collagen fragments claimed by applicants may potentially comprise non-homologous amino acid sequences, and that applicants specification "requires" homology among the claimed proteins in order to assure antiangiogenic activity. Being one skilled in the art and very familiar with the structure and function of collagen molecules, I respectfully disagree with the Examiner's conclusion that homology must be present in light of applicants' teachings and description in the specification. Although amino acid sequence homology among proteins can be a predictor of similar functions, it is not the only aspect that should be considered when making this assessment. Given applicants' teaching of NC1 domains, their location and methods for identifying antiangiogenic activity, together with the level of knowledge of those skilled in the art at the time of applicants' priority date, a skilled artisan could reasonably identify potential antiangiogenic fragments from C-terminal NC1 regions of collagen proteins without relying on information concerning amino acid sequences or homology to previously identified antiangiogenic fragments.

As discussed above, the most conserved regions of collagen molecules consist of the collagenous regions that are structurally identified by the prominent triple helical alpha chains. The areas that appear between the collagenous regions and at the terminal portions are identified as noncollagenous regions. Certainly some of the noncollagenous regions have homologous amino acid sequences, but given that "collagens comprise a large family of *genetically* distinct, but *structurally* related proteins", it is not surprising that much of the genetic diversity is observed in the noncollagenous regions. The lack of homology among the collagen molecules as discussed by the Examiner, therefore, actually supports the novelty and patentability of applicants' invention. Given the disparity in sequences of certain collagen molecules and



in the absence of the teachings in applicants' specification, one would not expect the noncollagenous regions of such molecules to have similar activity; more particularly, it would not be obvious based on sequence identification that the molecules would have comparable effects on angiogenesis. However, despite the disparity in sequences, based on the knowledge of collagen molecule structure and function together with applicants' teaching regarding NC1 region fragments, one skilled in the art could identify potential antiangiogenic fragments from collagen proteins. Applicants' novel discovery provides that specific domains of molecules, possibly having disparate sequence identities, have similar functions.

6. Applicants have defined the claimed invention in terms of structure (C-terminal NC1 domains) and function. The common functional feature shared by all members of this novel genus is the ability to inhibit angiogenesis. Applicants' groundbreaking discovery is that proteins comprising a genus of fragments of the C-terminal non-collagenous region of a collagen protein are antiangiogenic and can be used for the treatment of angiogenesis mediated diseases including cancer. Page 14, lines 19-23 of applicants' specification, defines cancer as angiogenesis-dependent cancers and tumors. As stated above, it is known that all members of the collagen family were structurally related and had non-collagenous domains at their C-terminal ends. Examples 1-3 teach how to isolate antiangiogenic fragments from this region. The specification further provides a method for evaluating antiangiogenic activity using assays such as the CAM assay (page 40, lines 1-21, of the applicants' specification). Therefore, it is my opinion that one of ordinary skill in the art would be able to isolate antiangiogenic fragments from any collagen, test for antiangiogenic activity, and detect the presence of endostatin proteins with an endostatin protein-specific binding antibody using the teachings of applicants' patent application.

7. Based on the foregoing, I believe that the applicants have sufficiently enabled the claimed invention. One skilled in the art could readily identify fragments from the C-terminal NC1 regions of collagen molecules, assess them for antiangiogenic activity according to the methods and techniques provided in applicants' specification.

8. I understand that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements the like are punishable by fine or imprisonment, or both under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date:

June 5, 2002

Bjorn R. Olsen  
BJORN R. OLSEN, PH.D.

Attorney Docket No. 05213-0331 (43170-273775)

## *Curriculum Vitae*

Name: Bjorn Reino Olsen

Address: 1053 Brush Hill Road  
Milton, MA 02186

Date of Birth: April 22, 1940

Place of Birth: Skien, Norway

### **Education:**

1967 Ph.D. University of Oslo, Norway

1967 M.D. University of Oslo Medical School, Norway

### **Research Fellowships:**

1963-1967 Research Assistant (Histology), Anatomical Institute, University of Oslo, Oslo, Norway

### **Academic Appointments:**

1967-1971 Assistant Professor, Anatomical Institute, University of Oslo, Oslo, Norway

1971-1975 Associate Professor, Anatomical Institute, University of Oslo, Oslo, Norway

1972-1976 Associate Professor, Department of Biochemistry, CMDNJ-Rutgers Medical School, Piscataway, NJ

1976-1985 Professor, Department of Biochemistry, CMDNJ-Rutgers Medical School, Piscataway, NJ

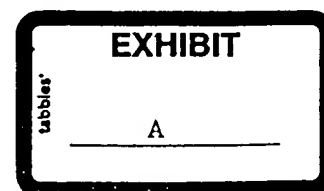
1985-1993 Hersey Professor of Anatomy, Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, MA

1990-1993 Chairman, Program in Cell and Developmental Biology, Harvard Medical School, Boston, MA

1993- Hersey Professor of Cell Biology, Department of Cell Biology, Harvard Medical School, Boston, MA

1996- Harvard-Forsyth Professor of Oral Biology, Harvard-Forsyth Department of Oral Biology, Harvard School of Dental Medicine, Boston, MA

1996- Chairman, Harvard-Forsyth Department of Oral Biology, Harvard School of Dental Medicine, Boston, MA



### **Other Professional Positions and Major Visiting Appointments:**

- |           |  |
|-----------|--|
| 1969-1970 | Army Service Researcher, Norwegian Defense Research Establishment, Division of Toxicology, Kjeller, Norway |
| 1971-1972 | Visiting Scientist, General Clinical Research Center, Philadelphia General Hospital, Philadelphia, PA      |

### **Awards and Honors:**

- |      |   |
|------|---|
| 1963 | Voss Award for Anatomical Research, University of Oslo Medical School   |
| 1974 | A. and K.E. Schreiner Award for Biological Research, Norwegian Academy of Sciences                                |
| 1983 | Co-chairman, Gordon Conference "Structural Macromolecules Collagen"   |
| 1984 | Co-chairman, New York Academy of Sciences Conference on "Biology, Chemistry and Pathology of Collagen"            |
| 1985 | Honorary A.M. Degree, Harvard University  |
| 1989 | Co-chairman, New York Academy of Sciences Conference on "Structure, Molecular Biology, and Pathology of Collagen" |
| 1992 | MERIT Award for scientific excellence, National Institutes of Health  |
| 1993 | MERIT Award for scientific excellence, National Institutes of Health  |
| 1995 | Co-chairman, New York Academy of Sciences, conference on "Molecular and Developmental Biology of Cartilage"       |
| 1995 | Humboldt Research Award for Senior U.S. Scientists  |
| 1996 | The Norwegian Academy of Sciences   |
| 1996 | President, International Society for Matrix Biology   |
| 2000 | Honorary Doctor of Science Degree, University of Medicine and Dentistry of New Jersey                             |
| 2000 | Honorary Doctor of Science Degree, University of Oslo, Norway   |
| 2001 | Distinguished Faculty Award, HSDM Distinguished Faculty Award   |

### **Major Committee Assignments:**

#### **National Committees:**

- |           |   |
|-----------|---|
| 1975-1976 | Ad Hoc Peer Reviewer, Molecular Cytology Study Section, NIH |
|-----------|---|

1976-1980	Member, Molecular Cytology Study Section, NIH
1978-	Ad Hoc Peer Reviewer, Initial Review Groups, NIH
1982	Co-organizer, NICHD Workshop "Normal and Abnormal Limb Development", NIH
1983-1987	Board of Scientific Counselors, NIDR, NIH
1986-2002	Scientific Advisory Board, Organogenesis, Inc., Canton, MA
1986	Program Committee, American Society for Cell Biology
1990-1991	Scientific Advisory Board, Metra Biosystems, Inc., Palo Alto, CA
1991-1992	Scientific Advisory Board, OsteoArthritis Sciences, Inc., Edison, NJ
1992-1996	Scientific Advisory Board, OsteoArthritis Sciences, Inc., Cambridge, MA
1994-2002	Board of Directors, Organogenesis, Inc., Canton, MA
1995-1998	National Arthritis and Musculoskeletal and Skin Diseases Advisory Council, National Institutes of Health, Bethesda, MD
2000-	Scientific Advisory Board, Prochon Biotech, Ltd., Israel
2001-	Scientific Advisory Board, National Marfan Foundation

#### **University of Oslo:**

1967-1971	Director, Laboratory for Molecular Anatomy, Anatomical Institute
1970	Member, University of Tromso Planning Committee

#### **Rutgers Medical School:**

1975-1978	Committee of Review
1978	Acting Chairman, Department of Biochemistry
1979-1984	Institutional Biosafety Committee
1980-1981	Pathology Chairman Search Committee
1981-1982	Microbiology Chairman Search Committee
1983-1985	Chairman, Institutional Radiation Safety Committee
1983-1985	Biotechnology Center Planning Committee
1985	Research Committee

#### **Harvard Medical School:**

Subcommittee of Professors

Faculty Council

Chairman - Ad Hoc Evaluation Committee for Professor of Orthopaedic Surgery at Massachusetts General Hospital

Faculty Council - Docket Committee

Member - Faculty of Arts and Sciences

Dunham Lectureship Committee

Soma Weiss Undergraduate Assembly Committee

Advisory Board - Child Health Research Center

M.D.-Ph.D. Admissions Committee

Ad Hoc Search Committee for Professor of Orthopaedic Surgery at Brigham and Women's Hospital

Chairman - Preclinical Promotions Board

Academic Review Committee

Standing Committee on Student Prizes and Awards in the Faculty of Medicine

Standing Committee on Conflicts of Interests and Commitment

Ad Hoc Search Committee for Professor of Cellular and Molecular Physiology at HMS/Professor of Medicine at Beth Israel Hospital

Scientific Advisory Board - Cutaneous Biology Research Center

Scientific Advisory Board - Massachusetts General Hospital

Chairman - Advisory Committee, Graduate Program in Biological and Biomedical Sciences

### **Editorial Boards:**

1981-1985 1991-1994 1998-	Journal of Cell Biology
1985-1991	Developmental Biology, Associate Editor
1986-1994	Calcified Tissue International
1986-1994	Development
1989-2002	Matrix, Matrix Biology, Associate Editor

2002-	Matrix Biology, Editor-in-Chief
1991-	Molecular Biology of the Cell (formerly Cell Regulation)
1991-1998	Developmental Dynamics, Associate Editor
1995-	Current Opinion in Cell Biology
1996-2001	Journal of Biological Chemistry

#### **Memberships:**

American Chemical Society  
 American Association for the Advancement of Science  
 American Association of University Professors  
 The American Society for Cell Biology  
 The American Society of Biological Chemists  
 American Association of Anatomists  
 East Coast Connective Tissue Society  
 International Society for Matrix Biology

#### **Major Research Interests:**

1. The role of extracellular matrix in embryonic development, particularly skeletal and vascular morphogenesis
2. Skeletal cell and molecular biology, molecular genetics and molecular pathology
3. Structure, biosynthesis and function of collagens

#### **Teaching Experience:**

1963-1967	Anatomy and physiology courses, Norwegian State College of Nursing, Oslo
1967-1971	Histology courses, University of Oslo Medical School
1972-1973	Biochemistry course for first year medical students, Rutgers Medical School
1974-1975	Histology and gross anatomy courses for first year medical students, University of Oslo Medical School
1975-1985	Biochemistry course for first year medical students, Rutgers Medical School
	Course in recombinant DNA techniques and participation in course in electron microscopy techniques, for graduate students at Rutgers Medical School

- 1985                    Histology course for first year medical students, Harvard Medical School
- 1985-                    Lecturer, graduate student courses in Cell Biology and Developmental Biology, Harvard Medical School
- 1986                    Tutor, New Pathway course "The Body" for first year medical students, Harvard Medical School
- 1988-1990              Senior Tutor, Cannon Society, Harvard Medical School

### **Teaching Awards:**

- 1978-79,                Best Lecturer of the First Year Class, Student Association,  
1980-81                Rutgers Medical School
- 1981-1982              Foundation of UMDNJ's Excellence in Teaching Award

### **Bibliography:**

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2.     Olsen BR. Electron microscope studies on collagen. II. Mechanism of linear polymerization of tropocollagen molecules. *Z Zellforsch* 1963; 59:199-213.
3.     Olsen BR. Electron microscope studies on collagen. III. Tryptic digestion of tropocollagen macromolecules. *Z Zellforsch* 1964; 61:913-919.
4.     Olsen BR. Electron microscope studies on collagen. IV. Structure of vitrosin fibrils and interaction properties of vitrosin molecules. *J Ultrastruct Res* 1965; 13:172-191.
5.     Dahl HA, Jansen J, Olsen BR. *Menneskets anatomi og fysiologi* [Anatomy and physiology textbook]. Oslo: JW Cappelen A/S, 1966.
6.     Olsen BR. Electron microscope studies on collagen. V. The structure of segment-long-spacing aggregates consisting of molecules renatured from isolated a-fractions of rat tail tendon collagen. *J Ultrastruct Res* 1967; 19:432-445.
7.     Olsen BR. Electron microscope studies on collagen. VI. The structure of segment-long-spacing aggregates consisting of molecules renatured from isolated a-fractions of codfish skin collagen. *J Ultrastruct Res* 1967; 19:446-473.
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9.     Froholm LO, Olsen BR. Electron microscopy of transfer RNA. *J Mol Biol* 1969; 40:305-306.
10.    Froholm LO, Olsen BR. A conformational model of serine transfer RNA proposed on the basis of electron microscopy. *FEBS Lett* 1969; 3:182-184.
11.    Laszlo F, Olsen BR. Cyanogen bromide peptides from the  $\alpha$ -1 chain of cod skin collagen. *Eur J Biochem* 1969; 11:140-147.



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13. Olsen BR, Svenneby G, Kvamme E, Tveit B, Eskeland T. Formation and ultrastructure of enzymically active polymers of pig renal glutaminase. *J Mol Biol* 1970; 52:239-245.
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# Collagens

## Overview of the family

The collagens constitute a superfamily of extracellular matrix proteins with a structural role as their primary function. Based on the exon structure of their genes as well as the configuration of the sequence domains of the proteins, they can be divided into several families or groups. Within each family, several homologous genes encode polypeptides that have domains with similar sequences. All collagenous proteins have domains with a triple-helical conformation. Such domains are formed by three subunits ( $\alpha$  chains), each containing a  $(\text{Gly-X-Y})_n$  repetitive sequence motif.

The presence of domains with a triple-helical molecular conformation (Fig. 1) provides collagens with regions of rigid, rod-like molecular structures.<sup>1,2</sup> In fibrillar collagens and short chain collagens each collagen molecule (after complete proteolytic removal of amino and carboxyl propeptides) contains only one such domain which accounts for almost the entire length of the molecule. In other collagens, such as FACIT collagens, basement membrane collagens, multiplexins, and collagens with transmembrane domains – MACITs, several short triple-helical domains are separated by non-triple-helical sequences.

Within triple-helical domains, each  $\alpha$  chain is coiled into an extended left-handed polyproline II helix and three  $\alpha$  chains are in turn twisted into a right-handed superhelix. The high resolution crystal structure of a triple-helical collagen-like peptide shows that the triple helix is surrounded by a cylinder of hydration; an extensive network of hydrogen bonds between water molecules and peptide acceptor groups stabilizes the structure.<sup>2</sup> Residues of 4-hydroxyproline in the Y-position of the  $(\text{Gly-X-Y})_n$  repeat sequence play a critical role in the hydrogen-bonded structure. The post-translational hydroxylation of collagen polypeptides therefore causes a significant increase in the thermal denaturation (melting) temperature of collagen triple helices. The triple-helical conformation requires a close packing of every third residue in each  $\alpha$  chain along the triple-helical axis and

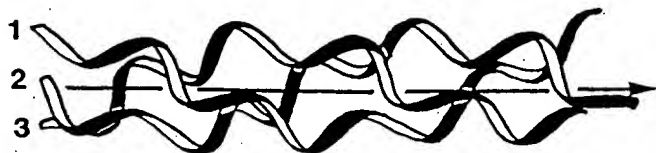


Figure 1. Molecular structure of the triple-helical conformation; three left-handed helices form a right-handed superhelix.

only glycol residues can be accommodated in this position. This explains why collagen mutations in which such triple-helical glycol residues are replaced by residues with more bulky side chains can cause severe abnormalities. Even replacement with an alanine residue can result in a local untwisting of the triple helix and an alteration in the characteristic hydrogen bonding pattern.<sup>2</sup>

Collagenous proteins usually form supramolecular aggregates (fibrils, filaments, or networks), either alone or in conjunction with other extracellular matrix components. Their major function is to contribute to the structural integrity of the extracellular matrix, or to help anchor cells to the matrix. Some of the non-fibrillar collagen types appear also to have important regulatory functions.

Based on detailed analyses of the exon structures of genes that encode collagenous proteins, a comparison of protein domains, and functional considerations, the collagen superfamily can be divided into several subfamilies, as follows.

1. *Fibrillar collagens*. This group includes types I, II, III, V, and XI collagen, with molecules forming banded (cross-striated) fibrils in various tissues.
2. *FACIT collagens*. These include types IX, XII, XIV, XVI, and XIX collagens, with molecules that are associated with fibrils formed by fibrillar collagens.
3. *Short chain collagens*. These include types VIII and X collagen, with short, dumb-bell shaped molecules that form part of unique networks in basement membrane regions (type VIII) and hypertrophic cartilage (type X).
4. *Basement membrane collagens*. These include several different molecules collectively known as type IV collagens. They represent the major collagenous components of basement membranes.
5. *Multiplexins*. These are molecules with multiple short triple-helical domains that are found mostly in basement membrane regions. Types XV and XVIII collagen currently belong to this group.
6. *Collagens with transmembrane domains – MACITs*. Types XIII and XVII collagen are cell-surface molecules with multiple extracellular triple-helical domains, connected to a cytoplasmic region by a transmembrane segment. Their orientation (with the carboxyl end in the extracellular space) is similar to that of other cell surface molecules with triple-helical domains, such as the type I macrophage scavenger receptor,<sup>3</sup> possibly

**Table 1** Chromosomal location of collagen genes

Gene locus	Chain designation	Chromosomal location in humans	Refs
<b>Fibrillar collagens</b>			
COL1A1	$\alpha 1(I)$	17q21.3-q22	17
COL1A2	$\alpha 2(I)$	17q21.3-q22	18
COL2A1	$\alpha 1(II)$	12q13-q14	19, 20
COL3A1	$\alpha 1(III)$	2q24.3-q31.17	19
COL5A1	$\alpha 1(V)$	9q34	21
COL5A2	$\alpha 2(V)$	2q24.3-q31	22
COL5A3	$\alpha 3(V)$		
COL11A1	$\alpha 1(XI)$	1p21	23
COL11A2	$\alpha 2(XI)$	6p212	24
<b>FACIT collagens</b>			
COL9A1	$\alpha 1(IX)$	6q12-q14	25, 26
COL9A2	$\alpha 2(IX)$	1p32.3-p33	27
COL9A3	$\alpha 3(IX)$	20q13.3	28
COL12A1	$\alpha 1(XII)$	6q12-q14	29
COL14A1	$\alpha 1(XIV)$	8q23	30
COL16A1	$\alpha 1(XVI)$	1p34-p35	31
COL19A1	$\alpha 1(XIX)$	6q12-q14	29
<b>Short chain collagens</b>			
COL8A1	$\alpha 1(VIII)$	3q11.1-q13.2	32
COL8A2	$\alpha 2(VIII)$	1p32.3-p34.3	33
COL10A1	$\alpha 1(X)$	6q21-q22	34
<b>Basement membrane collagens</b>			
COL4A1	$\alpha 1(IV)$	13q33-q34	35-37
COL4A2	$\alpha 2(IV)$	13q33-q34	36, 38, 39
COL4A3	$\alpha 3(IV)$	2q36-q37	40
COL4A4	$\alpha 4(IV)$	2q35-2q37.1	41, 42
COL4A5	$\alpha 5(IV)$	Xq22	43
COL4A6	$\alpha 6(IV)$	Xq22	44, 45
<b>Multiplexins</b>			
COL15A1	$\alpha 1(XV)$	9q21-q22	46
COL18A1	$\alpha 1(XVIII)$	21q22.3	47
<b>Collagens with transmembrane domains-MACITs</b>			
COL13A1	$\alpha 1(XIII)$	10q22	48
COL17A1	$\alpha 1(XVII)$	10q24.3	49
<b>Other collagens</b>			
COL6A1	$\alpha 1(VI)$	21q22.3	50-52
COL6A2	$\alpha 2(VI)$	21q22.3	50, 51
COL6A3	$\alpha 3(VI)$	2q37	50, 53
COL7A1	$\alpha 1(VII)$	3p21.1-p21.3	54, 55

the B-chain of the C1q complex,<sup>4,5</sup> and the macrophage protein MARCO.<sup>6</sup> The last three molecules have not traditionally been included among the collagens, but given their triple-helical domains<sup>7</sup> they can, with good justification, be described as collagen types. In fact, several proteins with triple-helical domains are not included among the collagens, most likely because they were not discovered in a 'collagen laboratory'. These include the collectins<sup>8,9</sup> (lung surfactant protein A, lung surfactant protein D, bovine conglutinin, collectin-43, and mannose binding protein), ficolins,<sup>10,11</sup> hibernation proteins,<sup>12</sup> the asymmetric form of acetylcholinesterase,<sup>13,14</sup> the subunits of C1q,<sup>15</sup> and a component of the inner ear.<sup>16</sup>

7. *Other collagens.* This group includes molecules (types VI and VII) that form specialized structures in a variety of tissues (e.g. microfibrils for type VI, anchoring fibrils for type VII).

Most collagen genes have now been cloned and their chromosomal locations determined. The recently described collagen types were found through molecular cloning; additional collagenous molecules are likely to be discovered through analyses of ESTs and genome sequences. The very large number of sequence entries in the GenBank/EMBL data bank makes it impractical to list the appropriate accession numbers in the pages that follow. The reader is instead referred to original articles for specific information. The chromosomal locations of

the human genes are given in Table 1. Useful databases for all collagens are Online Mendelian Inheritance in Man (OMIM) which can be accessed at <http://www3.ncbi.nlm.nih.gov/Omim/> and allied resources and links.

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## Fibrillar collagens

Collagen types I, II, III, V, and XI participate in the formation of fibrils with molecules packed in quarter-staggered arrays. Encoded by homologous, multiexon genes, they evolved to provide multicellular organisms, from sponges to humans, with supramolecular scaffolds for mechanical support and the proper environment for cellular migration, attachment and differentiation. Fibrillar collagens are synthesized as precursors, procollagens, that are proteolytically processed to collagen in the extracellular space.

Fibrillar collagens include five different molecular types (I, II, III, V, and XI) containing polypeptide subunits ( $\alpha$  chains), encoded by nine distinct genes.<sup>1-6</sup> The molecules are either homotrimers with  $\alpha$  chains of the same kind (types II and III) or heterotrimers with two or three different  $\alpha$  chains (types I, V, and XI). Some  $\alpha$  chains participate in the formation of more than one collagen type. For example, the product of the *COL2A1* gene,  $\alpha 1(\text{II})$ , forms homotrimeric type II collagen molecules and participates, as  $\alpha 3(\text{XI})$ , in the formation of heterotrimeric type XI collagen molecules. Also,  $\alpha 1(\text{XI})$  chains appear both in heterotrimeric type XI molecules in cartilage as well as in a bone variant of type V collagen, where it replaces the  $\alpha 1(\text{V})$  chain.<sup>7</sup> Finally,  $\alpha 2(\text{V})$  chains are found both in heterotrimeric type V molecules and in the vitreous form of type XI.<sup>8</sup> Because of their great similarity and ability to form mixed heterotrimers, types V and XI collagen are frequently referred to as type V/XI collagen.<sup>9</sup>

Each fibrillar collagen  $\alpha$  chain contains over 300 repeats of the triplet sequence -Gly-X-Y-, flanked by short non-triplet-containing sequences, telopeptides, at each end. About 50 per cent of the prolyl residues in the Y positions of the triplet domain are post-translationally converted to 4-hydroxyproline by the enzyme prolyl 4-hydroxylase (EC 1.14.11.2) located in the rough endoplasmic reticulum.<sup>10</sup> The active enzyme is a tetramer of two non-identical subunits,  $\alpha$  and  $\beta$ . The  $\beta$  subunit is the enzyme protein disulphide isomerase, and the tetramer prolyl 4-hydroxylase has disulphide isomerase activity.<sup>11</sup> In addition to prolyl hydroxylation, some lysyl residues in the Y position are hydroxylated by lysyl hydroxylase,<sup>12</sup> and the sequential action of galactosyl hydroxylase and glucosylgalactosyl hydroxylase adds mono- and disaccharides to some hydroxyllysyl residues.<sup>13</sup>

The  $\alpha$  chains of fibrillar collagens are synthesized as pro- $\alpha$  chains, with amino (N) and carboxyl (C) propeptides flanking the central (Gly-X-Y)<sub>n</sub>-containing domain (Fig. 1). Folding of a trimeric C-propeptide domain is the first step in intracellular assembly of homo- or heterotrimeric procollagen molecules; the chain composition of collagen molecules is therefore determined by the specificity by which C-propeptides of various procollagens interact. The folding of the triple-helical domain proceeds from the carboxyl end towards the amino end of the trimeric molecule in a zipper-like fashion and with a rate that is limited by *cis-trans* isomerization of peptidyl prolyl bonds.<sup>4</sup> Since prolyl and lysyl hydroxylases do not work on triple-helical substrates,<sup>10,12</sup> and the thermal stability of the triple helix depends on the level of hydroxylation of prolyl residues, the folding of the triple helix limits the degree of post-translational hydroxylation to what is needed for a stable triple helix at 37°C.<sup>5</sup> Substitutions of glycine residues in the Gly-X-Y repeats lower the stability, may lead to overmodification, and a decrease in the rate of secretion from cells resulting in intracellular retention and degradation.<sup>14</sup>

During extracellular processing of procollagen to collagen, the propeptides are removed from the major collagen triple-helical domain by specific endoproteases.<sup>13</sup> Of great interest is the recent finding that the C proteinase is identical to BMP-1, the mammalian homologue of the *Drosophila* tolloid gene product.<sup>15</sup> In *Drosophila* the tolloid protease is involved in processing the precursor of the BMP-2/4-like product of the *decapentaplegic* (*dpp*) gene; BMP-1 may likewise activate latent forms of BMPs or other members of the TGF- $\beta$  family of molecules. Controlled cleavage of the N-propeptide domain plays a role in fibrillogenesis and is thought to be important for regulation of fibril diameters.<sup>4,16,17</sup>

### ■ Purification and recombinant synthesis

To purify fibrillar collagens from tissues, pepsin has been commonly used to dissociate collagen triple-helical domains (these are pepsin resistant) from other extracellular matrix molecules. Repeated differential salt precipitations at neutral pH as well as in acid conditions are



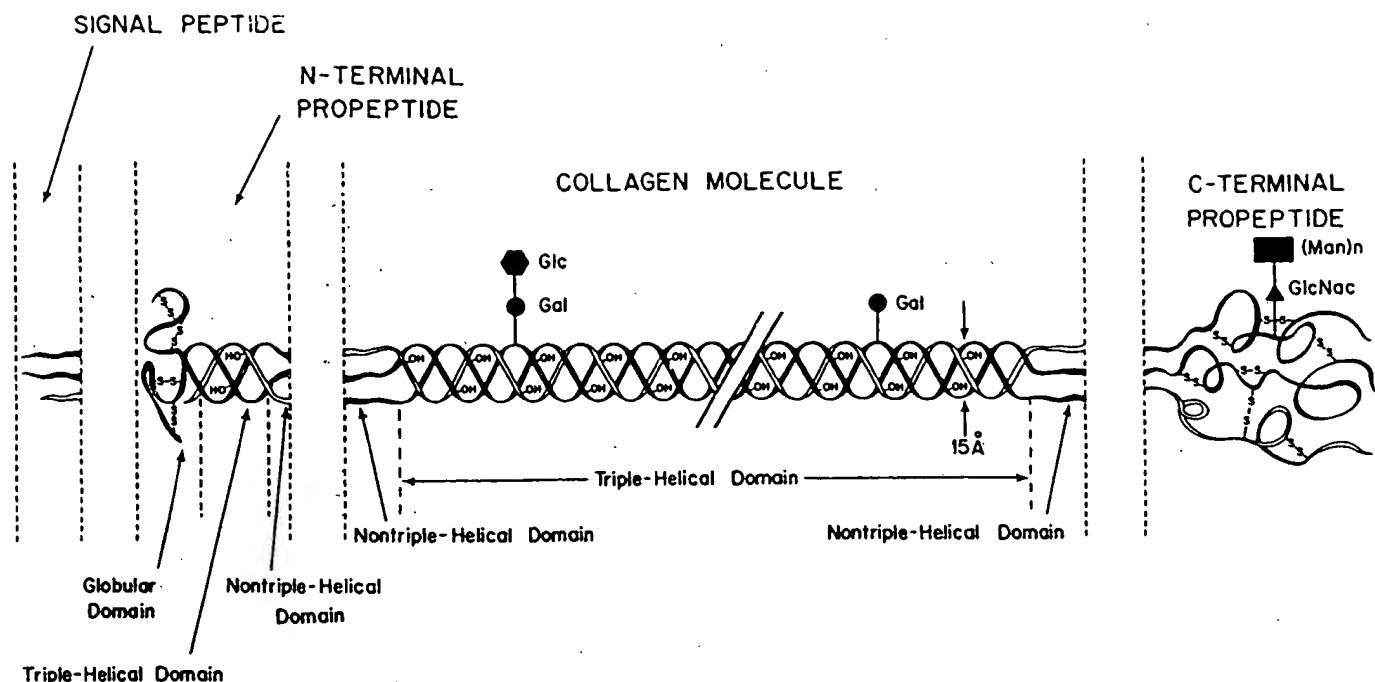


Figure 1. Diagram of the domains of a fibrillar procollagen molecule (type I). (From Olsen 1991.<sup>15</sup>)

then used to purify each fibrillar collagen type.<sup>18</sup> Types II, V, and XI collagen require higher salt concentrations than types III and I to precipitate.<sup>19</sup> Fibrillar procollagen molecules have been purified from media of cultured cells.<sup>20,21</sup> Addition of protease inhibitors and avoidance of acidic pH prevent the action of endogenous proteolytic enzymes that remove the propeptides, resulting in the isolation of intact precursor molecules. Purified collagens are available from a number of commercial sources. A good listing of many suppliers is provided in the BioSupplyNet Source Book, available at <http://www.biosupplynet.com>. Recently, recombinant fibrillar procollagens have been synthesized in mammalian cells as well as in insect cells transfected with genes encoding the  $\alpha$  and  $\beta$  subunits of prolyl 4-hydroxylase to ensure proper hydroxylation of prolyl residues.<sup>22-25</sup> A fragment of human type III collagen has been produced in *Saccharomyces cerevisiae*.<sup>72</sup>

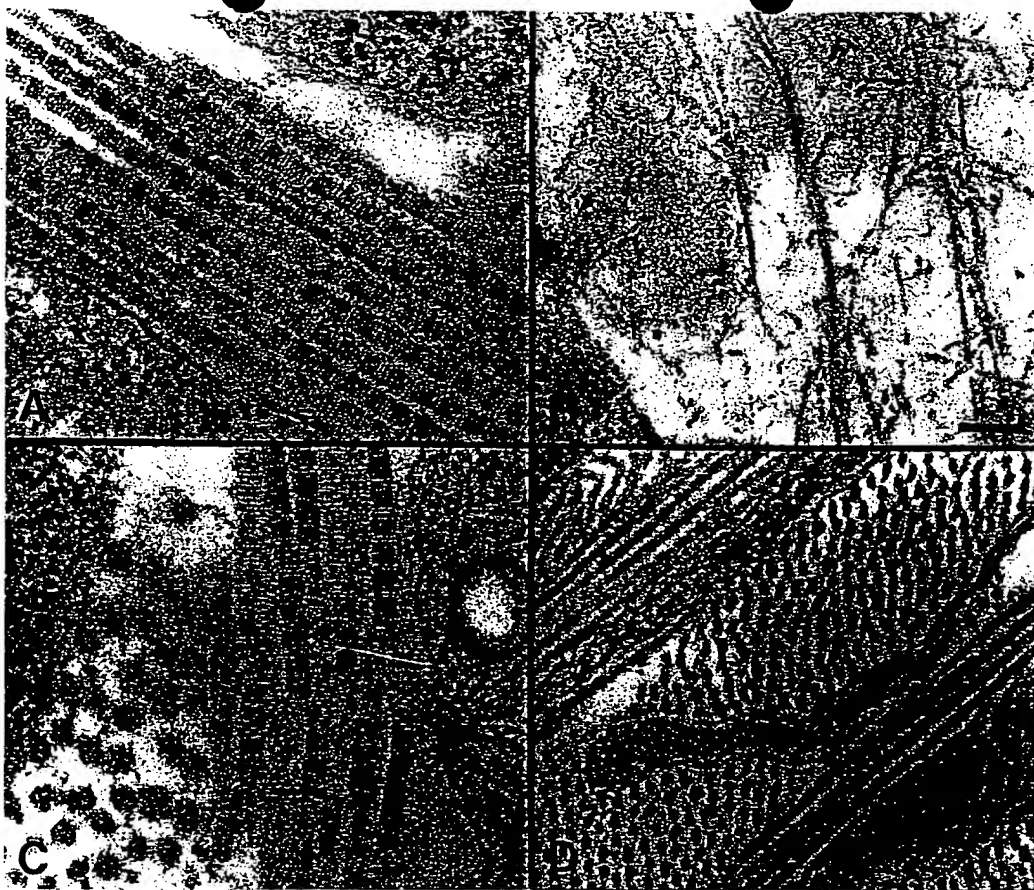
## Antibodies

Polyclonal and monoclonal antibodies against all fibrillar collagens from a number of animal species are available. Some of these antibodies are directed against epitopes in the propeptide domains of the procollagens;<sup>20</sup> others are directed against epitopes in the triple-helical domain.<sup>26,27</sup> Several monoclonal antibodies have been used for epitope mapping in conjunction with rotary shadowing and electron microscopy.<sup>27,28</sup> A variety of antibodies are available from commercial sources; a good listing of suppliers can be found in the BioSupplyNet Source Book.

## Activities

The triple-helical products of procollagen processing, fibrillar collagens, polymerize to form fibrils that serve as stabilizing scaffolds in extracellular matrices.<sup>29</sup> Within the fibrils, the 300 nm long rod-like molecules overlap with their ends by about 30 nm and are arranged in quarter-staggered arrays. The fibrils therefore have a periodic structure. Each period is 67 nm long and consists of a 'hole' zone with more loosely packed molecules and an overlap zone with more densely packed molecules. These zones can easily be visualized by negative staining and electron microscopy. When fibrils are positively stained, a periodic cross-striation pattern is observed, reflecting the distribution of clusters of charged amino acid residues along the collagen molecules.<sup>29</sup> Cell differentiation and migration during development are influenced by fibrillar collagens, and collagens interact with cells through integrin receptors on cell surfaces.

Collagen fibrils usually contain more than one type of collagen,<sup>30</sup> and such heterotypic fibrils are arranged in different patterns in different tissues; parallel fibril bundles in tendon, criss-crossing layers in cornea, and spiral arrangements in lamellar bone (Fig. 2). Heterotypic fibrils containing types I, III, and/or V collagens are expressed in a number of tissues of mesenchymal origin such as skin, tendon, ligaments, and bone, whereas fibrils with types II and XI are found predominantly in hyaline cartilage and the vitreous body of the eye. It is believed that the presence of small amounts of collagens V and XI within the fibrils limits fibril diameters due to steric hindrance, based on the incomplete removal of N-propeptides from types V and XI mole-



**Figure 2.** Collagen fibrils in 14-day chick embryo tendon (A), sternal cartilage (B), dermis (C), and corneal stroma (D). (Courtesy of Dr David Birk.)

cules.<sup>17,30,31</sup> Thus, the diameter of heterotypic collagen fibrils depends on the ratio between collagens V and I (or XI and II); the higher the ratio, the thinner the fibrils (Plate 11). Fibril properties are also dependent on interactions with FACIT collagens and small proteoglycans (decorin, biglycan, fibromodulin).<sup>4,32-39</sup> The ability of the C propeptide to serve as a ligand for the integrin  $\alpha 2 \beta 1$  may play a role in regulating fibrillogenesis at the cell surface.<sup>40,41</sup> Binding of fibrillar collagen to fibronectin may play a role in assembly of fibronectin fibres.<sup>42</sup>

## ■ Genes

The chicken  $\alpha 2(I)$  collagen gene was the first fibrillar collagen gene to be isolated and completely characterized.<sup>43,44</sup> Since then, cDNAs and genomic clones have been isolated for almost all fibrillar collagen genes, from a number of species. The number and the size of exons are similar in the various fibrillar collagen genes.<sup>2,3,45</sup> It is likely that many of the triple-helical domain exons evolved by repeated duplications of an exon unit of 54 base pairs. Alternative splicing generates transcripts encoding fibrillar procollagens with different N-propeptide domains in the *COL2A1*, *COL11A1*, and *COL11A2* genes.<sup>46-52</sup> Since the number of entries in the GenBank/EMBL data bank is very large, readers are referred to

original publications for accession numbers of specific sequences.

## ■ Mutant phenotypes/disease states

### Mutations in mice

Homozygous *Mov13* mice, carrying an insertion of proviral sequences in a transcriptional enhancer within the first intron of *Col1a1*, are developmentally arrested between days 11 and 12 of gestation due to a block of transcription of the gene in fibroblasts.<sup>53</sup> Heterozygous *Mov13* animals survive to adulthood and serve as models for the mild dominant form of osteogenesis imperfecta.<sup>54</sup> A frame-shift mutation in the C-propeptide coding domain of the *Col1a2* gene in the *oim* mouse also results in an osteogenesis imperfecta-like phenotype.<sup>55</sup> Disproportionate micromelia (*Dmm*) in mice is caused by a three-nucleotide deletion in the C-propeptide coding region of *Col2a1*,<sup>56</sup> while autosomal recessive chondrodysplasia (*cho*) is caused by a frame-shift mutation in *Col11a1* leading to loss of synthesis of  $\alpha 1(XI)$  collagen chains.<sup>57</sup>

Transgenic mice expressing dominant-negative mutant constructs have been generated for *Col1a1*, *Col2a1*, and *Col5a2*.<sup>58-63</sup> Mice with overexpression of wild-type gene or carrying inactivated ('knock out') alleles have also been described for several fibrillar collagen genes.<sup>64-66</sup>

## Human diseases

Mutations in *COL1A1* and *COL1A2*, the genes encoding the  $\alpha$  chains of type I procollagen, account for the majority of cases with osteogenesis imperfecta and for certain types of the Ehlers–Danlos syndrome.<sup>14,67–68</sup> Mutations in *COL3A1* cause Ehlers–Danlos syndrome type III and type IV. Mutations in *COL5A1* have been described in patients with Ehlers–Danlos syndrome type I and type II.<sup>69</sup> Mutations in *COL2A1* cause a spectrum of chondrodysplasias, including achondrogenesis II, hypochondrogenesis, spondyloepiphyseal dysplasia, and Kniest and Stickler syndromes.<sup>14</sup>

## Websites

A mutation database for the *COL1A1*, *COL1A2*, and *COL3A1* genes has been described<sup>70</sup> and is accessible at <http://www.le.ac.uk/genetics/collagen/collagen.html>. A review of almost 300 mutations in *COL1A1*, *COL2A1*, and *COL3A1*, as well as other collagen genes has been published.<sup>71</sup> The OMIM database ([www3.ncbi.nlm.nih.gov/Omim/](http://www3.ncbi.nlm.nih.gov/Omim/)) is an excellent resource for all fibrillar collagens and their associated inherited diseases.

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## FACIT collagens

FACIT collagens are a group of proteins that may serve as molecular bridges between fibrillar collagens and other extracellular matrix components. Their structure is strikingly different from that of other collagens in that their molecules contain two, three, or more relatively short triple-helical domains connected by non-triple-helical sequences. For some FACIT collagens, utilization of alternative promoters and alternative splicing give rise to different transcripts that are expressed in tissue-specific and time-dependent manners during embryonic development.

The FACIT (fibril associated collagens with interrupted triple helices) group of collagens includes at least five types of molecules, IX, XII, XIV, XVI, and XIX, composed of seven distinct polypeptide chains.<sup>1,2</sup> cDNA clones encoding an additional chain are currently being charac-

terized (M. Gordon; personal communication) so it is likely that the group will prove to contain even more types of collagens. The domain structure of the first molecule of this group to be described, type IX collagen, was predicted by cloning and sequencing of a cDNA encoding the chicken  $\alpha 1(\text{IX})$  chain.<sup>3</sup> Type IX molecules are heterotrimers consisting of  $\alpha 1(\text{IX})$ ,  $\alpha 2(\text{IX})$ , and  $\alpha 3(\text{IX})$  chains.<sup>4,5</sup> As shown in Fig. 1, they contain three triple-helical (COL) domains interrupted by non-triple-helical (NC) regions. Most of the NC domains contain cysteinyl residues forming disulphide bridges between subunits. One of the subunits,  $\alpha 2(\text{IX})$ , serves as a proteoglycan core protein and contains a glycosaminoglycan side chain attached to a seryl residue in the NC3 domain.<sup>6-10</sup> Before the structure of collagen IX was established by cDNA cloning/sequencing, it was, in fact, isolated as a proteo-

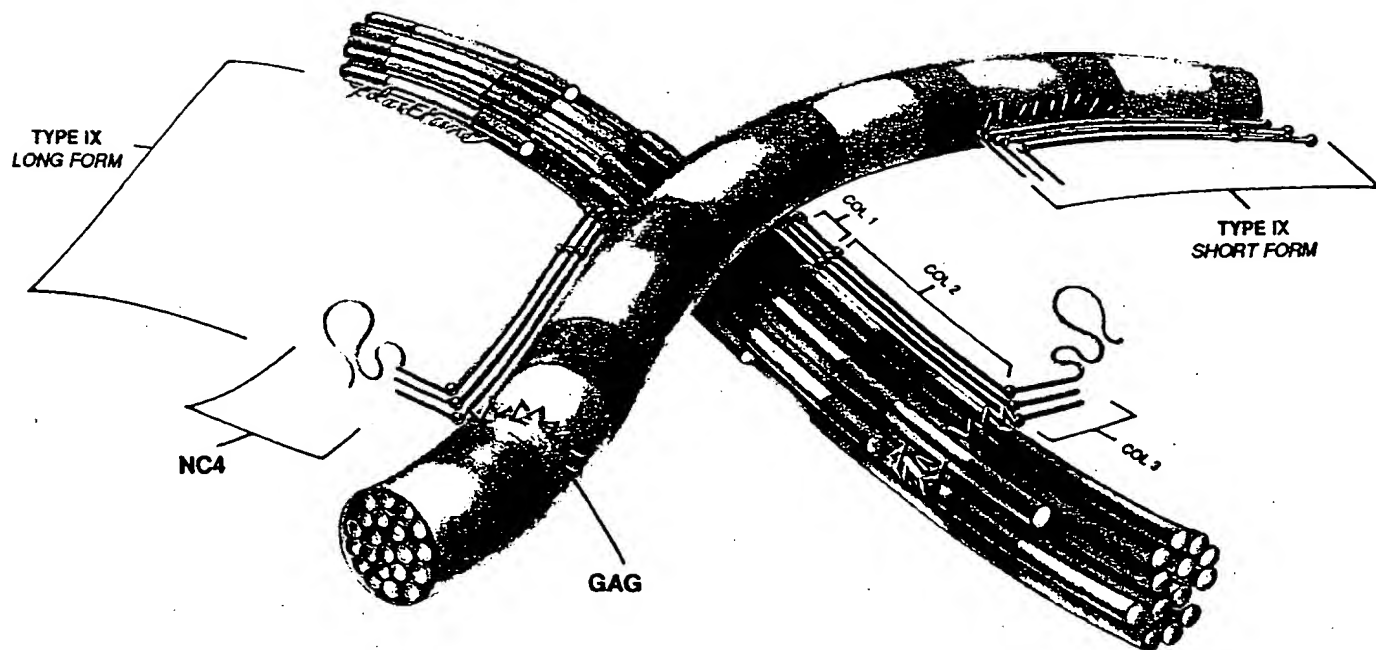


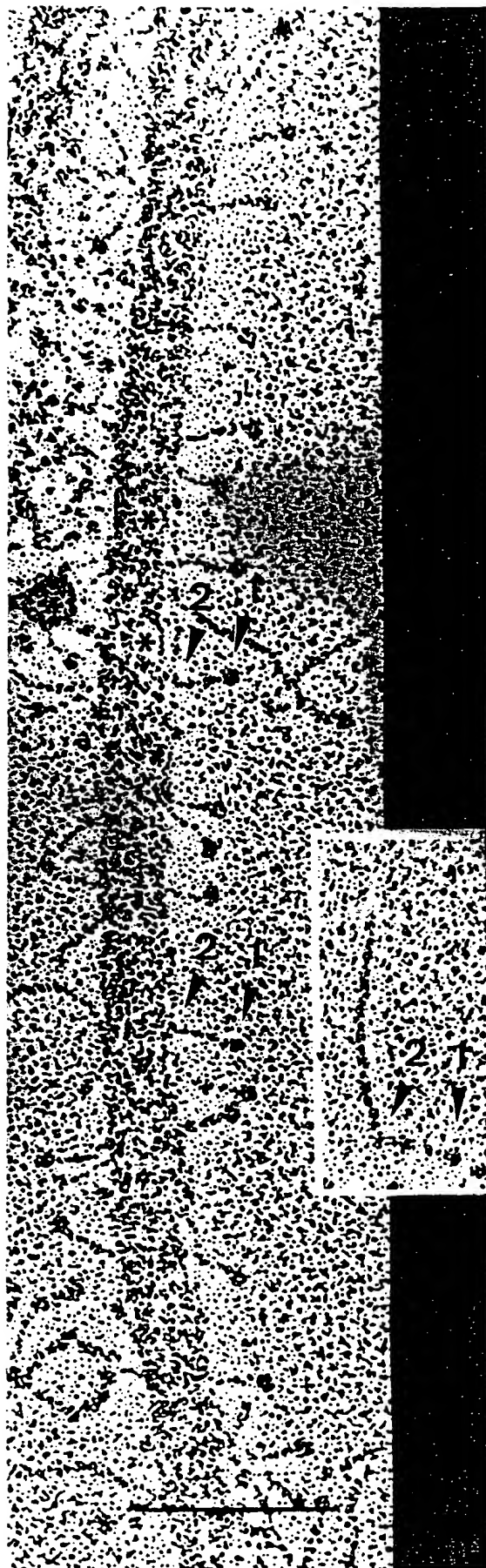
Figure 1. Diagram of type II collagen-containing fibrils with type IX collagen molecules on the surface. (From Jacenko *et al.* 1991.<sup>85</sup>)

glycan called PG-Lt from chicken cartilage.<sup>11,12</sup> In cartilage, the glycosylation of the  $\alpha 2(\text{IX})$  chain is incomplete and the glycosaminoglycan side chain is relatively short.<sup>13,14</sup> In the chicken vitreous body of the eye, however, the side chain is much longer and here type IX collagen may function primarily as a proteoglycan core protein.<sup>15,16</sup>

Type IX collagen molecules, expressed in hyaline cartilage, are associated with the surface of collagen fibrils such that two of the triple-helical domains are located at or close to the fibril surface, while an N-terminal globular domain is located in the perifibrillar space at the tip of a triple-helical arm (Fig. 2).<sup>17</sup> The type IX collagen molecules have an antiparallel orientation relative to the collagen II molecules within the fibrils. This has been deduced from the positions of covalent, hydroxypyridinium cross-links between the two types of molecules.<sup>18,19</sup> There are also covalent cross-links between different type IX collagen molecules, suggesting that type IX molecules on the surface of one fibril may be cross-linked to molecules on the surface of another fibril at points of intersection.<sup>19,20</sup> Immunofluorescence, *in situ* hybridization, and biochemical studies have shown that this FACIT collagen is also present in embryonic chick cornea and in the vitreous body.<sup>21</sup> Different tissues contain different forms of type IX molecules. These forms are translation products of two distinct mRNAs generated by alternative transcription of the  $\alpha 1(\text{IX})$  collagen gene.<sup>22</sup> In chondrocytes, the majority of the  $\alpha 1(\text{IX})$  transcripts are synthesized from an upstream transcription start site leading to the formation of mRNA that codes for a polypeptide with an N-terminal, globular domain.<sup>23–25</sup> In embryonic chick cornea,<sup>22,26,27</sup> the vitreous body,<sup>15,16,27</sup>

neural retina,<sup>28,29</sup> perinotochordal matrix,<sup>30,31</sup> and early limb buds,<sup>32</sup> the majority of the transcripts are synthesized from a downstream, alternative start site, leading to the formation of mRNA encoding an  $\alpha 1(\text{IX})$  with an alternative signal peptide sequence and lacking the N-terminal globular domain.

Types XII and XIV collagen are homologous, but distinct, homotrimeric molecules.<sup>33–41</sup> Their subunits contain two triple-helical (COL) domains separated by an NC region of more than 40 amino acid residues, a relatively short (<100 amino acid residues) non-triple-helical C-terminal region, and a very large (>1500 amino acid residues) N-terminal non-triple-helical domain<sup>2</sup> (Fig. 3). Within the native molecules the COL domains of the three subunits form a triple-helical tail attached to a central globule from which three non-triple-helical arms or finger-like structures project.<sup>40–42</sup> The central globule and the arms are composed of the N-terminal NC domains. For both types XII and XIV collagen, alternative splicing of primary transcripts generates molecular diversity. Two major molecular forms of type XII collagen differ in the lengths of the N-terminal NC (NC3) domains.<sup>2</sup> In form XIIA, the NC3 domains contain 18 fibronectin type III repeats and four von Willebrand factor A-like domains.<sup>34</sup> In the shorter form XIIB there are 10 fibronectin type III repeats and two von Willebrand factor A-like domains<sup>35</sup> (Fig. 3). Both forms contain identical signal peptides and are encoded by mRNAs with identical 5' untranslated sequences. For form XIIB, two variants generated by alternative splicing at the 3' end of the primary transcript have been described.<sup>43</sup> In variant XIIB1 the carboxyl non-triple-helical domain NC1 is 74 amino acid residues long and contains an acidic region



followed by a basic region. In variant XIIB2 the NC1 domain is much shorter (only 19 residues) and lacks these features.<sup>43</sup>

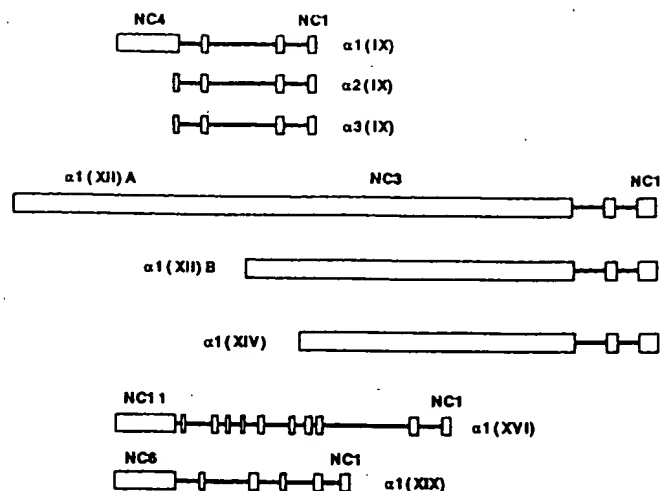
In type XIV collagen the NC3 domain is somewhat smaller than in collagen XIIB. Splice variations affecting the 5' untranslated region of type XIV collagen mRNA, the N-terminal fibronectin type III repeat as well as the 3' region have been described.<sup>38,39,44</sup> Undulin, a protein isolated from human placenta, has been shown to be encoded by the type XIV collagen gene and represents one of these variants.<sup>44,45</sup> The initially isolated undulin was a protein composed of only von Willebrand factor A-like domains and fibronectin type III repeats and no collagen sequences, but this was probably caused by proteolysis during isolation of the protein.<sup>46</sup>

Types XII and XIV collagen are found in most dense connective tissues. There is considerable overlap between their tissue distributions, but there are also some differences.<sup>47-49</sup> In bovine skin, type XII collagen is particularly concentrated in the papillary dermis, while type XIV collagen is present in the reticular dermis.<sup>40</sup> In periosteum, type XIV collagen appears restricted to the outer fibrous layer while type XII collagen is expressed both in this layer and in the innermost layer of osteogenic cells.<sup>50</sup> Antibodies to both collagens show labelling along type I-containing fibrils.<sup>51</sup>

The structure of types XVI and XIX collagens has been deduced from cDNA sequences. Both molecules contain multiple triple-helical and non-triple-helical domains. Type XVI collagen contains 10 triple-helical domains interspersed with 11 short non-triple-helical sequences<sup>52,53</sup> (Fig. 3). The C-terminal triple-helical domain (COL1) shows structural homology with the COL1 domains of types IX, XII, and XIV collagens. The non-triple-helical domains contain multiple cysteine residues that are arranged in a pattern similar to that found in cuticle collagens of *C. elegans*.<sup>54</sup> Type XVI collagen cDNAs were initially isolated from human fibroblast<sup>52</sup> and placental cDNA libraries,<sup>53</sup> but subsequent studies have shown a wide range of expressing tissues.<sup>55</sup> Type XIX collagen was originally discovered through cDNA cloning with RNA from the human rhabdomyosarcoma cell line RD (CCL136).<sup>56</sup> The predicted polypeptide was found to contain five triple-helical (COL) domains, interspersed with and flanked by six non-triple-helical (NC) domains (Fig. 3). The coding region is relatively small in comparison to the size of the transcript due to a long 3'-UTR (5 kb). The  $\alpha 1(XIX)$  gene is located on human chromosome 6q12-q13, syntenic to the  $\alpha 1(IX)$  and  $\alpha 1(XII)$  genes.<sup>57</sup> In mouse embryos the  $\alpha 1(XIX)$  gene is transcribed in many organs but only a few adult tissues such as brain, eye and testis appear to express this collagen.<sup>58</sup>

Figure 2. Rotary shadowing micrograph of type II collagen fibril with type IX molecules on the surface. (From Vaughan *et al.* 1988.<sup>17</sup>)





**Figure 3.** Diagram showing the domain structures of the members of the FACIT group of proteins. The domains are counted from the C terminus. Non-triple-helical domains are shown as open rectangles; triple-helical domains as solid lines.

## ■ Purification and recombinant synthesis

Type IX collagen can be purified from the medium of chondrocyte cultures or from cartilage tissue extracts.<sup>59</sup> Triple-helical fragments of the molecule have been purified from pepsin extracts of cartilage.<sup>60</sup> Types XII and XIV collagens have been purified from neutral salt extracts of skin and tendons<sup>40</sup> and as triple-helical fragments by pepsin extraction.<sup>36</sup> Type XVI collagen has not been purified as a protein from tissues, but a 160–210 kDa protein was detected by polyclonal antibodies in Western blots, consistent with the predicted structure from cDNA.<sup>53,55</sup> A recombinant  $\alpha 1(\text{XIX})$  peptide was produced in *E. coli*.<sup>58</sup>

## ■ Antibodies

A large number of antibodies against FACIT collagens are available. Polyclonal antibodies against synthetic peptides deduced from nucleotide sequences<sup>7</sup> and polyclonal<sup>20</sup> as well as monoclonal antibodies<sup>61–63</sup> against protein fragments have been described for type IX collagen. A monoclonal antibody against a synthetic peptide derived from cDNA sequences recognizes the chicken  $\alpha 1(\text{XII})$  chain by Western blotting, and has been used for immunohistochemical studies.<sup>47</sup> Monoclonal antibodies against bovine type XII (TL-A) and type XIV (TL-B) collagen are also available.<sup>40,51</sup> Polyclonal antibodies have been made against a synthetic peptide and a recombinant fragment of type XVI collagen.<sup>55</sup>

Polyclonal antibody against a recombinant  $\alpha 1(\text{XIX})$  peptide was raised and used for Western blotting.<sup>58</sup>

## ■ Activities

Type IX collagen molecules are arranged in a periodic fashion along heterotypic collagen II/XI fibrils.<sup>17</sup> Covalent

lysine-derived hydroxypyridinium cross-links between types IX and II molecules, as well as between collagen IX molecules, stabilize the fibril association.<sup>18,19</sup> This arrangement suggests that type IX collagen may serve as a molecular bridge between fibrils as well as between fibrils and other extracellular matrix constituents. Based on the colocalization of types XII and XIV with collagen I-containing fibrils in tissues, and the partial sequence similarity between types IX, XII, and XIV collagen, it is thought that types XII and XIV collagen associate with type I collagen fibrils in a similar fashion to type IX with type II fibrils.<sup>2,64</sup> Although type XIV collagen molecules did not bind to type I collagen in experiments with isolated matrix molecules,<sup>65</sup> it has been shown that type XII collagen can become incorporated into type I collagen fibrils when it is present during fibril formation; removal of the triple-helical domains of type XII reduced its ability to polymerize with type I collagen.<sup>66</sup> Both types XII and XIV collagen can bind to the dermatan sulphate chains of the fibril-associated proteoglycan decorin.<sup>65,67</sup> The two FACIT molecules may therefore interact with fibrils both directly and indirectly and may be important in keeping fibrils together in bundles, or alternatively, in preventing fibril fusion during tissue morphogenesis. Addition of types XII and XIV collagen to gels of type I collagen promoted fibroblast-induced gel contraction.<sup>68</sup> The effect was lost upon denaturation of the proteins, but was not reduced when the triple-helical domains were digested with bacterial collagenase. Since type XIIA carry glycosaminoglycan side chains while XIIB does not,<sup>41</sup> it is possible that cells can regulate the hydrophilic properties of perifibrillar compartments by controlling the expression of the two major splice variants of type XII collagen.

## ■ Genes

cDNA sequences are available for chicken  $\alpha 1(\text{IX})$ ,  $\alpha 2(\text{IX})$ , and  $\alpha 3(\text{IX})$ ,<sup>3,69–71</sup> mouse  $\alpha 1(\text{IX})$ ,  $\alpha 2(\text{IX})$ ,<sup>72,73</sup> and human  $\alpha 1(\text{IX})$ ,  $\alpha 2(\text{IX})$  and  $\alpha 3(\text{IX})$ .<sup>25,74,75</sup> Partial cDNA sequences from rat,<sup>76</sup> bovine,<sup>69</sup> and dog (GenBank L77390)  $\alpha 1(\text{IX})$  are also reported. Genomic clones and sequences are available for the chicken  $\alpha 1(\text{IX})$  and  $\alpha 2(\text{IX})$  genes<sup>23,69,77</sup> as well as the mouse and human  $\alpha 1(\text{IX})$  and  $\alpha 2(\text{IX})$  genes.<sup>9,24,73,75,78</sup> Genomic and cDNA sequences are also available for  $\alpha 1(\text{XII})$ ,  $\alpha 1(\text{XIV})$ ,  $\alpha 1(\text{XVI})$  and  $\alpha 1(\text{XIX})$  collagens from several species.<sup>33,34,38,39,52,53,56–58</sup>

## ■ Mutant phenotype/disease states

Evidence for a role of type IX collagen in maintaining long-term stability of cartilage comes from studies of transgenic mice and genetic abnormalities in humans. In transgenic mice expressing an  $\alpha 1(\text{IX})$  transgene with an in-frame deletion in the central triple-helical domain (COL2)<sup>80</sup> and in homozygous mice with inactivated  $\alpha 1(\text{IX})$  alleles,<sup>78</sup> articular cartilage developed degenerative changes resembling those of human osteoarthritis. In humans, demonstration of linkage between the COL9A2 locus and multiple epiphyseal dysplasia 2 (EDM2) (OMIM

600204)<sup>81</sup> was followed by confirmation of linkage in a second family with EDM2 and identification of a splice site mutation in COL9A2 causing exon skipping and deletion of 12 amino acid residues in the COL3 domain of the  $\alpha 2(\text{IX})$  collagen chain.<sup>82</sup> Affected individuals in the two families develop stiffness and pain in knees during childhood and adolescence. X-rays of knees reveal flattened, irregular epiphyses and gradually appearing osteoarthritis. Because of the heterotrimeric structure of type IX collagen molecules, one would expect mutations in COL9A1 and COL9A3 to also cause multiple epiphyseal dysplasia with early onset osteoarthritis.

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## Short chain collagens

Types VIII and X collagen, composed of the three chains  $\alpha 1(\text{VIII})$ ,  $\alpha 2(\text{VIII})$ , and  $\alpha 1(\text{X})$ , form the subgroup of short chain collagens, so named because their subunits are short (only about 60 kDa) as compared with fibrillar collagen chains. Despite similarities in domain structure, amino acid sequences, and genomic exon configurations, the two types show very different temporal and spatial expression. Given the similarity in exon structure it is likely that the three genes evolved by duplication of a common precursor gene.

Type VIII collagen was originally identified as a product of bovine aortic and rabbit corneal endothelial cells, but is also synthesized by non-vascular cells.<sup>1</sup> The molecule is probably a heterotrimer composed of  $\alpha 1(\text{VIII})$  and  $\alpha 2(\text{VIII})$  chains in a ratio of two to one,<sup>2</sup> but the existence of homotrimeric molecules composed entirely of  $\alpha 1(\text{VIII})$  or  $\alpha 2(\text{VIII})$  chains cannot be ruled out.<sup>3</sup>

Type X collagen is a specific product of hypertrophic chondrocytes and is a useful marker for chondrocyte maturation to hypertrophy.<sup>4</sup> Except for the avian eggshell,<sup>5</sup> it

does not appear to be expressed in other tissues outside hypertrophic cartilage. The molecule is a homotrimer of  $\alpha 1(X)$  chains and has a domain structure that is similar to that of type VIII collagen; a central triple-helical (COL1) domain of 50 kDa is flanked by N-terminal (NC2) and C-terminal (NC1) non-triple-helical domains.<sup>6</sup> Both type VIII and type X molecules appear as 130 nm long rods with knobs at both ends by electron microscopy after rotary shadowing.<sup>5,7</sup> The COL1 and NC1 domains of both types are encoded by one large exon, whereas the NC2 domain is encoded by a small additional exon.<sup>7-9</sup> Additional exons (one for  $\alpha 1(X)$  or two for  $\alpha 1(VIII)$ ) encode the 5' untranslated portion of the mRNA. This exon configuration is in stark contrast to the multiexon structure of most other collagen genes.

Despite the similarities, a distinct tissue distribution has been found for the two short chain collagens: type X is restricted to hypertrophic cartilage,<sup>4</sup> whereas type VIII is distributed in various tissues including Descemet's membrane, vascular subendothelial matrices, heart, liver, kidney, perichondrium, and lung, as well as several malignant tumours including astrocytoma, Ewing's sarcoma, and hepatocellular carcinoma.<sup>1,10,11</sup>

In Descemet's membrane, type VIII collagen molecules represent major components of a hexagonal lattice structure,<sup>12</sup> with type VIII molecules most probably linked by interactions involving the non-triple-helical end regions (Fig. 1). Type X collagen molecules may form the same kind of polymer in hypertrophic cartilage,<sup>13,14</sup> and colocalization with a proteoglycan epitope<sup>15</sup> suggests a complex with proteoglycans. The expression of type X collagen is regulated primarily at a transcriptional

level.<sup>16,17</sup> Evidence from 'knockout' and overexpression studies suggests that chondrocyte hypertrophy and type X collagen expression is negatively regulated by PTHrP and its receptor in growth plates.<sup>18</sup>

## ■ Purification and recombinant synthesis

The triple-helical domain of type VIII collagen can be purified from Descemet's membrane by pepsin extraction.<sup>1</sup> The digested material can be precipitated with NaCl at neutral pH, and purified further by chromatography through agarose and by reverse phase HPLC.<sup>1</sup> Intact type VIII collagen can be recovered from the medium of cultured endothelial cells.<sup>1</sup> Type X collagen can be isolated intact from the medium of chicken hypertrophic chondrocytes kept in long-term culture or as a triple-helical fragment by pepsin extraction of hypertrophic cartilage.<sup>4,19</sup> Site-directed mutagenesis of human type X collagen has been used to determine the role of each domain in molecular assembly and secretion.<sup>20</sup> Three mutants with mutations in the C-terminal NC1 domain that are similar to those found in patients with Schmid type metaphyseal chondrodysplasia (see below), were unable to assemble into homotrimers *in vitro* or *in vivo* and were not secreted from cells.<sup>20</sup> In-frame deletions within the triple-helical domain did not prevent molecular assembly and secretion of pepsin-resistant triple-helical molecules.<sup>20</sup>

## ■ Antibodies

Polyclonal antibodies against bovine type VIII collagen have been used for expression studies and immunoblots.<sup>11,21,22</sup> Monoclonal antibodies against the bovine  $\alpha 1(VIII)$  chain have been used for immunoelectron microscopy to demonstrate that the backbone within Descemet's membrane is composed of type VIII collagen.<sup>12</sup> These antibodies are commercially available. Polyclonal antibodies against sheep type VIII collagen have also been produced.<sup>10</sup> A conformation-dependent monoclonal antibody, X-AC9, against chicken type X collagen has been used extensively for investigations of the tissue distribution, ultrastructure, and thermal stability.<sup>4</sup> Several other antibodies against chicken, mouse, bovine, and human type X collagens are available.<sup>15,23-25</sup>

## ■ Activities

Type VIII collagen is the major constituent of the hexagonal lattice observed in Descemet's membrane, as demonstrated by immunoelectron microscopy.<sup>12</sup> It is possible that the general function of type VIII collagen is to provide an open, porous structure that can withstand compressive force. Type X collagen in hypertrophic cartilage may play a similar role by providing a scaffold to prevent local collapse as the hypertrophic cartilage matrix is removed during endochondral ossification.<sup>13,26,27</sup> Several observations suggest a positive and/or negative

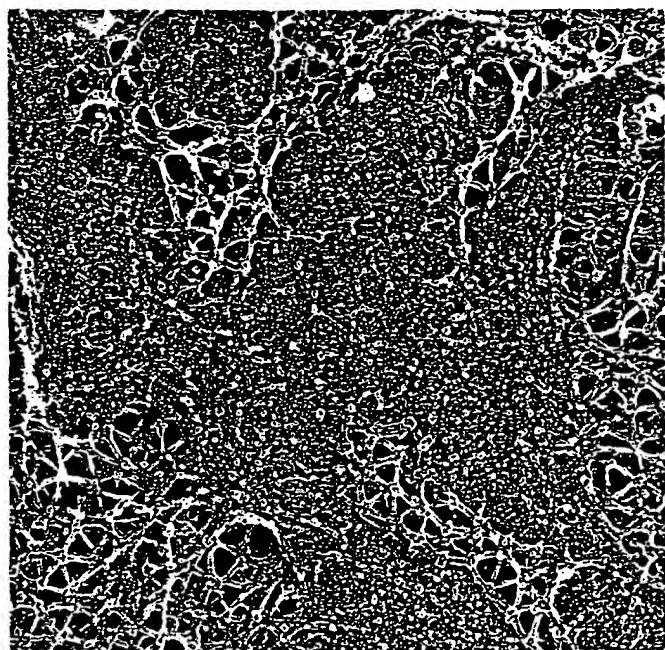


Figure 1. Hexagonal network structures formed in cultures of bovine corneal endothelial cells. The backbone of the network is composed of type VIII collagen molecules. (From Sawada, H. *et al.* 1984.<sup>46</sup>)

role for type X collagen in the calcification of hypertrophic cartilage.<sup>4</sup>

## ■ Genes

The primary structures of the  $\alpha 1(\text{VIII})$  and  $\alpha 2(\text{VIII})$  chains are strikingly similar to that of  $\alpha 1(\text{X})$  collagen. cDNA and genomic DNAs encoding rabbit, human, and mouse  $\alpha 1(\text{VIII})$  and  $\alpha 2(\text{VIII})$  chains have been isolated and characterized.<sup>3,6,7,28</sup> The human  $\alpha 1(\text{VIII})$  and  $\alpha 2(\text{VIII})$  genes are located on chromosomes 3 and 1, respectively.<sup>3,28</sup> The chicken type X gene was the first to be isolated among the short chain collagen genes.<sup>8,28</sup> The bovine,<sup>29</sup> mouse,<sup>30-32</sup> and human<sup>32-34</sup> type X genes were subsequently sequenced and characterized. The *COL10A1* gene has been localized to human chromosome 6q21-q22;<sup>35</sup> the mouse gene is on chromosome 10.<sup>33</sup>

## ■ Mutant phenotype/disease states

Mice carrying a transgene encoding an  $\alpha 1(\text{X})$  collagen chain with an in-frame deletion in the triple-helical domain developed skeletal abnormalities within 2-3 weeks after birth.<sup>36</sup> Histology showed a decrease in the width of the zone of hypertrophic chondrocytes in growth plates, decreased bone formation in the metaphyses of long bones, and bone marrow abnormalities.<sup>36</sup> Craniofacial abnormalities were also noted.<sup>37</sup> Although an initial study of type X collagen null mice reported no phenotypic abnormalities,<sup>38</sup> subsequent studies<sup>24</sup> of mice that are homozygous for inactivated *Col10a1* alleles show distinct growth plate abnormalities. Recent analyses (O. Jacenko, personal communication) indicate that these are similar to those seen in the type X collagen transgenics. These findings in mice are consistent with the demonstration that Schmid metaphyseal chondrodysplasia (OMIM 156500), an autosomal dominant disorder in humans with short stature and growth plate abnormalities, is caused by mutations in the *COL10A1* gene. Since the first discovery of a frame-shift-causing deletion in the C-terminal NC1 domain,<sup>39</sup> a large number of mutations in the NC1 domain of type X collagen have been found in patients with Schmid metaphyseal chondrodysplasia.<sup>40-44</sup> Except for one report describing mis-sense mutations in the N-terminal NC2 domain,<sup>45</sup> all mutations have been in the NC1 domain. Studies on the molecular assembly and secretion of mutant polypeptides<sup>20</sup> support the initial hypothesis<sup>39</sup> that Schmid metaphyseal chondrodysplasia is caused by haploinsufficiency for type X collagen.

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## Basement membrane collagens

Type IV collagen is the major collagenous component of basement membranes, forming a network structure with which other basement membrane components (laminin, nidogen, heparan sulphate proteoglycan) interact. Six distinct genes are identified as belonging to the type IV collagen gene family. They form three pairs of genes on three different chromosomes; within each pair the genes are arranged head-to-head and regulated by a bidirectional promoter.

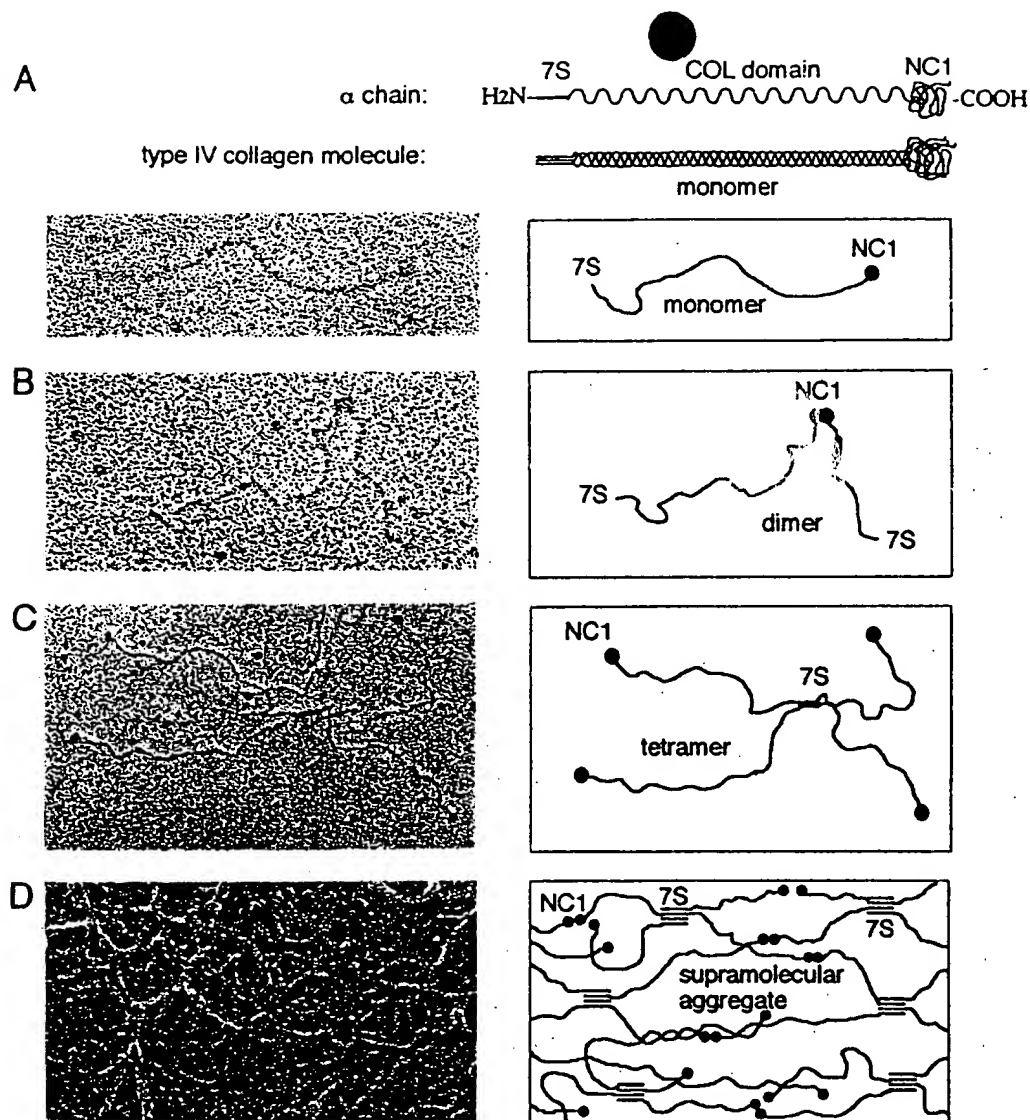
Collagen molecules, composed of two  $\alpha 1(\text{IV})$  and one  $\alpha 2(\text{IV})$  chain, have long been recognized as a major component of basement membranes.<sup>1–3</sup> Each of the two chains is about 1700 amino acid residues long and contains at least three distinct domains: an N-terminal cysteine-rich (7S) domain, a central triple-helical domain, and a C-terminal non-triple-helical domain (NC1).<sup>1</sup> Type IV molecules assemble into a network which is quite different from the banded fibrils formed by fibrillar collagen types (Fig. 1). Within the network, separate molecules are covalently cross-linked within laterally associated 7S domains and associated by end-to-end interactions through their NC1 domains.<sup>1,2,4</sup> Lateral associations between the triple-helical domains also contribute to the network structure<sup>1,2</sup> (Fig. 1).

While type IV collagen molecules, composed of  $\alpha 1(\text{IV})$  and  $\alpha 2(\text{IV})$  chains, are broadly expressed, molecules containing combinations of four additional chains,  $\alpha 3(\text{IV})$ – $\alpha 6(\text{IV})$ , are important components of specialized basement membranes.<sup>4–9</sup> In the kidney glomerular basement membrane, molecules of  $\alpha 1(\text{IV})$ ,  $\alpha 2(\text{IV})$  are replaced by  $\alpha 3(\text{IV})$ ,  $\alpha 4(\text{IV})$ , and  $\alpha 5(\text{IV})$  chains as development pro-

ceeds.<sup>10</sup> The  $\alpha 6(\text{IV})$  chain is present in epidermal basement membranes, around smooth muscle cells and adipocytes, and in Bowman's capsule and renal distal tubules, but absent from glomerular basement membranes.<sup>11</sup> The precise chain composition of triple-helical molecules assembled from the  $\alpha 3(\text{IV})$ – $\alpha 6(\text{IV})$  chains is not entirely clear, but it is believed that  $\alpha 3(\text{IV})$  and  $\alpha 4(\text{IV})$  chains form heterotrimeric molecules. Also, analyses of bovine seminiferous tubule basement membranes have established a structural linkage between  $\alpha 3(\text{IV})$  and  $\alpha 5(\text{IV})$  chains.<sup>12</sup>  $\alpha 3(\text{IV})/\alpha 4(\text{IV})$  molecules and molecules containing  $\alpha 5(\text{IV})$  chains may therefore be components of the same network. This helps to explain the observation that glomerular basement membranes from patients with Alport syndrome caused by mutations in  $\alpha 5(\text{IV})$  (see below) are defective in  $\alpha 3(\text{IV})$  and  $\alpha 4(\text{IV})$ .<sup>13,14</sup>

### ■ Purification and recombinant synthesis

Fragments of type IV collagen can be extracted from basement membranes with pepsin (resulting in triple-helical fragments) or with bacterial collagenase (resulting in non-triple-helical domains).<sup>1,15</sup> Intact type IV collagen composed of  $\alpha 1(\text{IV})$  and  $\alpha 2(\text{IV})$  chains can be isolated by acetic acid extraction of murine EHS-tumour tissue,<sup>1</sup> and is commercially available. Pepsinized material is also commercially available. The BioSupplyNet Source Book contains a good listing of suppliers. The NC1 domains of the human  $\alpha 1(\text{IV})$ – $\alpha 5(\text{IV})$  chains have been synthesized in *E. coli*<sup>16</sup> and in insect cells.<sup>17</sup>



**Figure 1.** Electron micrographs of type IV collagen monomer, dimer, tetramer, and supramolecular aggregate after rotary shadowing, and schematic illustrations of the structure, and supramolecular assembly of type IV collagen. (a) Each  $\alpha$  chain contributes to the 400 nm long triple-helical (COL) domain. This contains a number of interruptions in the Gly-X-Y-repeat sequence (not shown). A globular non-triple-helical domain (NC1) is located at the C-terminal end. The 7S domain is at the N-terminal end. Three  $\alpha$  chains form a triple-helical molecule. The triple-helical molecules are the building blocks (monomers) of the basement membrane meshwork. Monomers associate into dimers that are stabilized by disulphide bonds between NC1 domains (b) or tetramers that are stabilized by disulphide bonds between the N termini (c). The supramolecular network is formed by assembly of dimers, and tetramers, and strengthened by lateral associations between molecules (d). Other basement membrane components such as laminin, proteoglycans, and nidogen are incorporated into the type IV collagen meshwork. (Courtesy of Dr Eihiro Adachi, School of Medicine, Kitazato University.)

## ■ Antibodies

A variety of antibodies are available.<sup>1,10,18,19</sup> These include antibodies against the 7S and NC1 domains, as well as antibodies against pepsin fragments.<sup>1</sup> Both polyclonal and monoclonal antibodies against type IV collagen are commercially available from several sources. FITC-anti- $\alpha 5(\text{IV})$  and Texas red-anti- $\alpha 2(\text{IV})$  antibodies for diagnosis of Alport syndrome are commercially available; a good listing of suppliers can be found in the BioSupplyNet Source Book.

## ■ Activities

Type IV collagen can interact with cells indirectly through laminin. Strong binding of type IV collagen to laminin is mediated by nidogen/entactin,<sup>20,21</sup> a glycoprotein of about 150 kDa which binds tightly to laminin<sup>22,23</sup> and has binding sites also for type IV collagen and cells.<sup>3</sup> In addition, direct low affinity interaction between laminin and type IV collagen is possible.<sup>2,24</sup> Type IV collagen also binds to heparin and heparan sulphate proteoglycan<sup>2,25-27</sup> and heparin can inhibit type IV collagen polymerization.<sup>25</sup>

Many cell types adhere to type IV collagen,<sup>1,28</sup> and peptides from within type IV sequences can inhibit this adhesion<sup>29</sup>. A major cell binding site in  $\alpha1(IV)/\alpha2(IV)$  heterotrimers is localized about 100 nm from the N terminus of the molecule; this triple-helical binding site interacts with  $\alpha1\beta1$  and  $\alpha1\beta2$  integrins on cells.<sup>4</sup> Recombinant fibulin-2 has a weak affinity for type IV collagen, but binding of nidogen to immobilized fibulin-2 allowed the formation of ternary complexes with collagen IV.<sup>30</sup>

## Genes

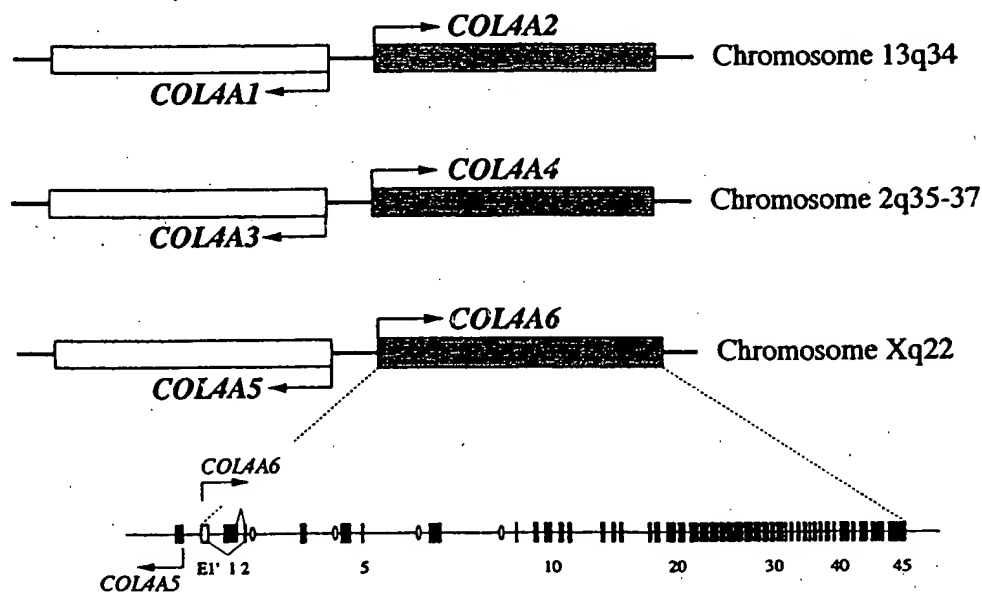
Six distinct type IV collagen genes have been identified. These are organized in three sets *COL4A1/COL4A2*, *COL4A3/COL4A4*, and *COL4A5/COL4A6* which in humans are localized on three different chromosomes, 13, 2, and X, respectively (Fig. 2).<sup>4</sup> Within each set the genes are arranged head-to-head and their expression is regulated by bidirectional promoters between the genes. The 5' ends of the genes overlap; the transcription start sites are separated only by 130 bp in human<sup>31,32</sup> and mouse<sup>33,34</sup>  $\alpha1(IV)$  and  $\alpha2(IV)$  genes. The transcriptional regulation of *COL4A1/COL4A2* is well characterized.<sup>35,36</sup> Transcription of *COL4A6* seems to be controlled by two alternative promoters.<sup>37</sup> The complete primary structures of mouse and human  $\alpha1(IV)$  and  $\alpha2(IV)$  chains have been deduced from cDNA sequences, and mouse and human genomic clones have been extensively characterized.<sup>38-41</sup> The human  $\alpha3(IV)$  and  $\alpha4(IV)$  genes, located on chromosome 2, have also been well characterized.<sup>42-44</sup> The primary structure of

the human  $\alpha5(IV)$  and  $\alpha6(IV)$  chains has been established by sequencing of cDNAs<sup>8,9</sup> and genomic clones.<sup>37,45,46</sup>

Type IV collagen genes have been characterized in several invertebrates, such as *Drosophila*,<sup>47</sup> *Caenorhabditis elegans*,<sup>48-51</sup> *Ascaris suum*,<sup>52</sup> and sea urchin, *Strongylocentrotus purpuratus*.<sup>53,54</sup> The protein encoded by the  $\alpha1(IV)$  collagen gene in *Drosophila* is quite similar to the vertebrate type IV collagen chains, but the gene has fewer exons and is smaller than the corresponding vertebrate gene.<sup>47</sup> In *C. elegans* the *clb-1* and *clb-2* genes are homologous to the vertebrate  $\alpha1(IV)$  and  $\alpha2(IV)$  collagen genes;<sup>48</sup> however, these genes are located on separate chromosomes. Interestingly, mutations in the  $\alpha1(IV)$  gene in *C. elegans* result in temperature-sensitive lethality during late embryogenesis.<sup>49</sup>

## Mutant phenotype/disease states

Homozygous, *Col4a3*-null mice show a phenotype that is similar to Alport syndrome in humans.<sup>55,56</sup> Decreased glomerular filtration leads to uraemia, changes in the glomerular basement membrane causes proteinuria, and glomerulonephritis develops. Histological and molecular analyses indicate that the absence of  $\alpha3(IV)$  chains causes loss of  $\alpha4(IV)$  and  $\alpha5(IV)$  chains from the glomerular basement membrane, and leads to increased levels of type VI collagen and perlecan, as well as retention of  $\alpha1(IV)$  and  $\alpha2(IV)$  chains.<sup>55,56</sup> Canine X-linked hereditary nephritis is an animal model of human X-linked Alport syndrome, and has been shown to be caused by a premature stop codon in the  $\alpha5(IV)$  collagen chain.<sup>57</sup>



**Figure 2.** Illustration of the organization, and chromosomal locations of the human type IV collagen genes. The genes coding for the six type IV collagen chains are located in pairs in a head-to-head manner on three different chromosomes. The genes are depicted as rectangles, and the flanking regions by horizontal lines. For the *COL4A6* gene, the locations of exons are indicated by vertical bars, and introns by horizontal lines. The exons are numbered from the 5' end of the gene. Introns of unknown sizes are indicated by ellipsoids. Note that the first two exons, E1' and 1, of the gene are alternatively utilized, and spliced to exon 2 as indicated by free lines.



Three different human diseases directly involve type IV collagen genes or their translation products. Goodpasture syndrome (OMIM 233450), an autoimmune disorder causing progressive glomerulonephritis and pulmonary haemorrhage, is caused by antibodies that bind to an antigen (the Goodpasture antigen) in basement membranes of kidney glomeruli and lung alveoli.<sup>58</sup> The Goodpasture antigen is the NC1 domain of  $\alpha3(IV)$  collagen chains.<sup>59,60</sup> Dimers of  $\alpha3(IV)$  NC1 domains, isolated from bovine kidney, can induce an autoimmune response in rabbits similar to Goodpasture syndrome.<sup>61</sup> Mutations in *COL4A5*, located on the X chromosome, have been demonstrated in more than 200 cases of X-linked Alport familial nephritis (OMIM 301050).<sup>62</sup> In cases of autosomal recessive Alport syndrome (OMIM 203780), mutations have been identified in the  $\alpha3(IV)$  and  $\alpha4(IV)$  genes.<sup>63</sup> In rare cases of diffuse leiomyomatosis associated with Alport syndrome, large deletions involving the  $\alpha5(IV)$  and  $\alpha6(IV)$  genes have been found.<sup>64</sup> Autosomal dominant benign familial haematuria (OMIM 141200), characterized by thinning of the glomerular basement membrane and normal renal function, has been linked to the *COL4A3/COL4A4* locus and shown to be caused by a mutation in *COL4A4*.<sup>65</sup>

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## Multiplexins

The non-fibrillar collagens type XV and type XVIII are broadly expressed in many tissues, but are present at particularly high levels in internal organs. They contain multiple short triple-helical domains, separated and flanked by non-triple-helical regions. Based on a considerable degree of similarity in some of their structural domains, they are classified as members of a novel subfamily of collagens called multiplexins.

The two members of this class of proteins, types XV and XVIII collagen, have been given the name multiplexins<sup>1</sup> because they both contain multiple-triple-helix domains with interruptions. They share considerable homology at the amino acid level, but are sufficiently different to rule out the possibility that they could form mixed heterotrimers like types V and XI fibrillar collagens.

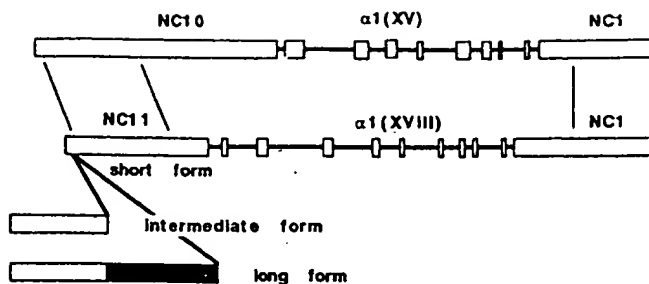
First isolated by cross-hybridization during screening of a placental cDNA library,<sup>2</sup> type XV collagen has now been completely characterized at the nucleotide level.<sup>3,4</sup>  $\alpha 1(XV)$  collagen chains contain nine triple-helical (COL) domains that are separated and flanked by non-triple-helical (NC) regions (Fig. 1). The N-terminal region (NC10\*) consists of 530 amino acid residues and is almost as large as the triple-helical region; the C-terminal non-

triple-helical region (NC1\*) is somewhat smaller (256 amino acid residues).

The  $\alpha 1(XVIII)$  collagen chain contains 10 triple-helical domains, separated and flanked by non-triple-helical sequences<sup>1,5</sup> (Fig. 1). A comparison with  $\alpha 1(XV)$  shows a striking similarity in size between the six most C-terminal triple-helical domains of the two collagens.<sup>1,6</sup> Also, at the amino acid level there is over 60 per cent identity between the carboxyl half of the 315 residue NC1 domain of  $\alpha 1(XVIII)$  and the corresponding portion of  $\alpha 1(XV)$ .<sup>7</sup> Both collagens contain four cysteinyl residues in this region and may therefore have a similar tertiary structure. Another region of homology is a 200 residue sequence at the amino end of the short variant (see

\* For type IV basement membrane collagens, FACIT collagens, and short-chain collagens the numbering of triple-helical and non-triple-helical domains starts by counting from the C terminus of the molecule. In keeping with this tradition, this numbering system has been used also for types XV<sup>3</sup> and XVIII<sup>1</sup> collagen and is followed here. Numbering the domains from the N terminus has been suggested for  $\alpha 1(XV)$  and  $\alpha 1(XVIII)$  collagens,<sup>5,7</sup> but serves only to create confusion and should be avoided.





**Figure 1.** Diagram showing the domain structures of types XV and XVIII collagen chains. Non-triple-helical domains are indicated by rectangles; triple-helical domains are indicated by a solid line. Thin vertical lines between the two chains delineate regions of homology in the NC10/NC11, and NC1 regions of the two chains. Three variant transcripts give rise to three different  $\alpha 1(XVIII)$  chains with different NC11 domains. These are indicated as the short, intermediate, and long forms. The frizzled-like region in the long form is indicated by the stippled rectangle.

below) of  $\alpha 1(XVIII)$  and the corresponding region of  $\alpha 1(XV)$ .<sup>3</sup> This sequence is 45 per cent identical between the two collagens and is homologous with a region of thrombospondin-1, the fibrillar procollagens V and XI, and members of the FACIT group.<sup>7</sup>

Northern blot analyses show that types XV and XVIII collagens are expressed in several major internal organs and in several cell types, including fibroblasts and endothelial cells.<sup>1,3,5,8</sup> There is considerable overlap between the expression patterns of the two collagens, but also distinct differences. For example, while both transcripts are found in the kidney,  $\alpha 1(XV)$  transcripts are low in lung and liver while those of  $\alpha 1(XVIII)$  are very high, particularly in liver. Immunohistochemical studies demonstrate a wide distribution of the two collagens, with a particular concentration in basement membrane regions.<sup>9,10</sup>

## ■ Purification and recombinant synthesis

A portion of the NC1 domain of type XV collagen has been expressed as a recombinant protein in bacteria and used for generation of specific polyclonal antisera.<sup>14</sup> Fragments of type XVIII collagen have been produced both in bacteria and in insect cells.<sup>15</sup>

## ■ Antibodies

Antibodies have been generated against both types XV and XVIII collagens and used for Western blotting and immunohistochemical studies.<sup>2,9,10,14</sup>

## ■ Activities

The supramolecular assemblies and functions of multiplexins have not been characterized. Of considerable

interest, however, is the finding that a 20 kDa angiogenesis inhibitor from a murine haemangioendothelioma, called endostatin, represents a fragment of the carboxyl region of the NC1 domain of  $\alpha 1(XVIII)$  collagen chains.<sup>15</sup> This portion of type XVIII collagen, produced as a recombinant peptide and injected into mice, causes nearly complete suppression of tumour-induced angiogenesis and tumour growth.<sup>15</sup>

## ■ Genes

Genomic and cDNA sequences for mouse and human  $\alpha 1(XV)$  and  $\alpha 1(XVIII)$  collagens are available.<sup>1-6,9,12-14,16</sup> Of interest is that the  $\alpha 1(XVIII)$  collagen gene contains two alternative promoters and that transcripts from one of these promoters can be alternatively spliced. This gives rise to three alternative  $\alpha 1(XVIII)$  transcripts that encode  $\alpha 1(XVIII)$  collagen chains with very different N-terminal (NC11) non-triple-helical domains<sup>9,12</sup> (Fig. 1). The shortest variant, transcribed from the most 5' promoter, contains mostly the thrombospondin-1 homology region. An intermediate-sized variant, transcribed from the most 3' promoter, contains a different signal peptide and an additional region of about 200 residues that is rich in acidic amino acid residues. The longest variant, also transcribed from the most 3' promoter, contains in addition a 250 residue region inserted between the acidic domain and the thrombospondin-1 homology region. This inserted region contains 10 cysteinyl residues and shows a striking similarity to the extracellular ligand-binding domain of frizzled receptors, with a frizzled-like distribution of the cysteines.<sup>9,12</sup>

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## Collagens with transmembrane domains – MACITs

Types XIII and XVII collagen are cell surface molecules with multiple extracellular triple-helical domains, connected to a cytoplasmic region by a transmembrane segment. They represent a new class of cellular adhesion molecules by which cells are connected to extracellular matrix. Type XIII collagen is expressed on the surface of fibroblasts, while type XVII collagen is a component of hemidesmosomes in epithelial cells.

Although the overall structures of types XIII and XVII collagen are quite different, it is reasonable to include them in a separate group of collagenous proteins, based on their membrane association. In analogy with the term FACIT for fibril-associated collagens, the type XIII/XVII group has therefore been designated the MACIT (membrane-associated collagens with interrupted triple-helices) group.<sup>1</sup> As discussed in the overview of the collagen superfamily (pp. 380–382), one can also include the macrophage scavenger receptors<sup>2</sup> and MARCO<sup>3</sup> in this group of proteins (Fig. 1).

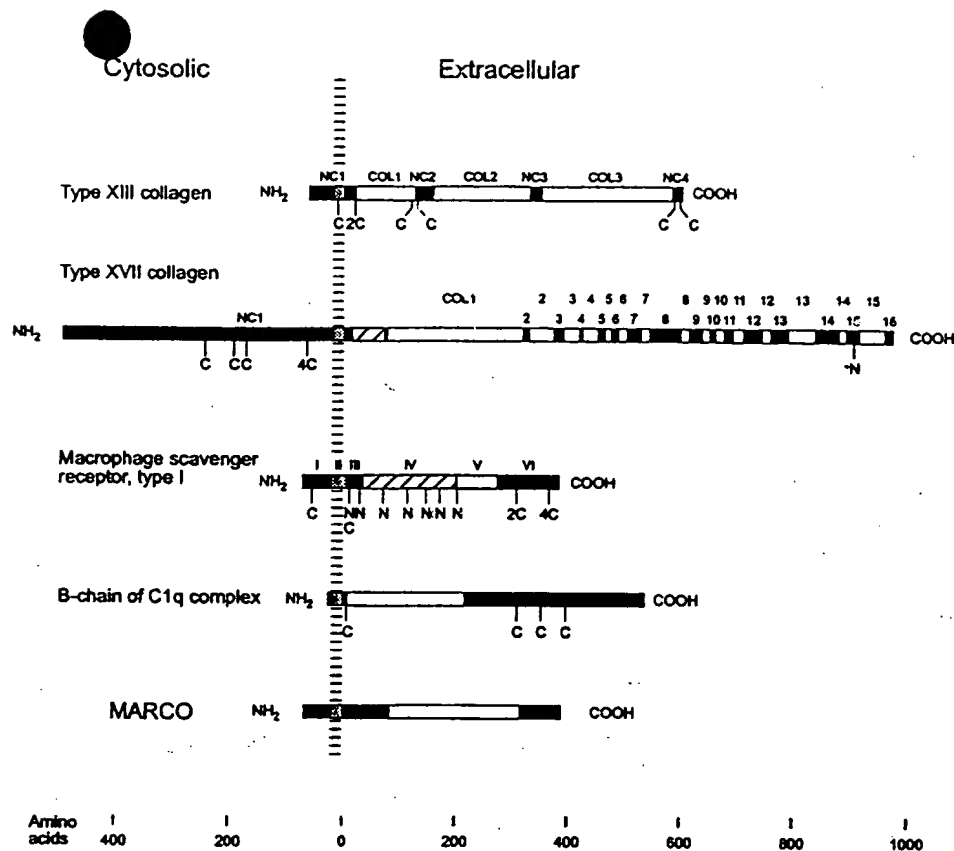
Type XIII collagen, initially identified by cross-hybridization during screening of a human cDNA library with a mouse type IV collagen probe,<sup>4</sup> is encoded by a gene that gives rise to a number of transcripts by alternative splicing.<sup>5</sup> These transcripts encode a polypeptide chain with three triple-helical domains, separated by non-triple-helical regions. In the different splice variants the length of the N- and C-terminal triple-helical domains varies considerably. It is believed that type XIII molecules are homotrimers; how the synthesis of the different variants can be reconciled with trimerization and the proper folding of triple-helical domains is not clear.<sup>1</sup> Type XIII collagen is widely expressed in human tissues and cell lines. Western blots of extracts of human HT-1080 fibrosarcoma cells show the presence of bands of expected size (about 67 and 54 kDa),<sup>4</sup> and these antibod-

ies show localization at discrete sites along the cell surface (Fig. 2). By *in situ* hybridization,  $\alpha 1(\text{XIII})$  transcripts have been found in epidermis and hair follicles, muscle, intestinal wall, cartilage, and bone.<sup>6</sup> In placenta, stromal cells of the villi, endothelial cells of developing capillaries, and cells of the cytotrophoblastic columns are all positive for type XIII collagen transcripts.<sup>7</sup>

Type XVII collagen is a component of hemidesmosomes and represents the autoantigen BPAG2, causing an acquired blistering skin disease, bullous pemphigoid.<sup>8,9</sup> Sequencing of chicken, mouse, and human  $\alpha 1(\text{XVII})$  collagen cDNA shows that it contains a large cytoplasmic N-terminal domain (almost 500 amino acid residues) with an extracellular triple-helical region consisting of eight heptad repeats, likely to form a coiled-coil trimer, and 15 short triple-helical domains separated by non-triple-helical regions.<sup>10–13</sup> Rotary shadowing of affinity-purified type XVII collagen isolated from bovine mammary gland epithelial cells showed a structure composed of a globular head, a central rod, and a flexible tail.<sup>14</sup> It is likely that the globular domain is the cytoplasmic region, the central rod is the heptad repeat region, and the flexible tail represents the interrupted triple-helical domain. Immunoelectron microscopy shows that type XVII collagen is a hemidesmosomal component with the extracellular domains localized in the anchoring filaments between the cell surface and the lamina densa of the underlying basement membrane.<sup>15</sup>

### ■ Purification and recombinant synthesis

Type XIII collagen has not been isolated as a protein from tissues, but type XVII has been isolated from primary human keratinocytes, HaCaT cells, and bovine corneal



**Figure 1.** Diagrams comparing the domain structures of membrane-associated polypeptides containing collagenous sequences. The numbering of non-triple-helical, and triple-helical domains is shown above the corresponding polypeptide. Filled rectangles indicate non-triple-helical domains; open rectangles indicate triple-helical domains. The plasma membrane is indicated by the vertical dashed line. C, cysteine residue; N, potential *N*-glycosylation site. The scale below is given in amino acid residues, counted from the transmembrane domain. (Modified from Pihlajaniemi and Rehn (1995),<sup>1</sup> courtesy of Dr T. Pihlajaniemi.)

epithelial cells.<sup>16–18</sup> Mouse Balb/K keratinocytes were transfected with a full length type XVII collagen cDNA and shown to assemble as a triple-helical homotrimer.<sup>17</sup> A portion of the extracellular domain of type XVII collagen has been expressed as a recombinant protein in insect cells.<sup>15</sup>

## ■ Antibodies

Antipeptide antibodies are being used to study the expression and cellular localization of type XIII collagen.<sup>1</sup> About half the sera from patients with bullous pemphigoid and most sera from patients with herpes gestationis contain autoantibodies against type XVII collagen. Monoclonal antibodies recognizing both the extracellular and intracellular domains are available and have been used for immunofluorescence, Western blotting, and immunoelectron microscopy.<sup>16</sup>

## ■ Activities

Type XIII collagen is expressed at focal adhesion sites in cultured fibroblasts and may therefore represent a matrix-binding anchoring molecule at such sites (Fig. 2).

Type XVII is part of the multiprotein hemidesmosome complex that mediates adhesion of epithelial cells to the underlying basement membrane.<sup>19</sup> Transfection experiments with various mutant cDNAs suggest that the localization of type XVII collagen in the hemidesmosome is mediated by the cytoplasmic domain and requires interaction with sequences in the cytoplasmic domain of the  $\beta 4$  integrin subunit.<sup>20</sup>

## ■ Genes

cDNA and genomic clones for mouse and human  $\alpha 1$ (XIII) collagen are available.<sup>21–24</sup> Alternative splicing gives rise to multiple transcripts of 2.5–2.8 kb.<sup>5,25</sup> For type XVII collagen cloning of chicken, mouse, and human cDNAs have been reported.<sup>10–13</sup> The entire human *COL17A1* gene has also been characterized.<sup>26</sup>

## ■ Mutant phenotype/disease states

No mutations in type XIII collagen are known. In contrast, several mutations in *COL17A1* have been demonstrated in patients with generalized atrophic benign epidermolysis bullosa (OMIM 226650).<sup>27–29</sup> This is a rare non-lethal

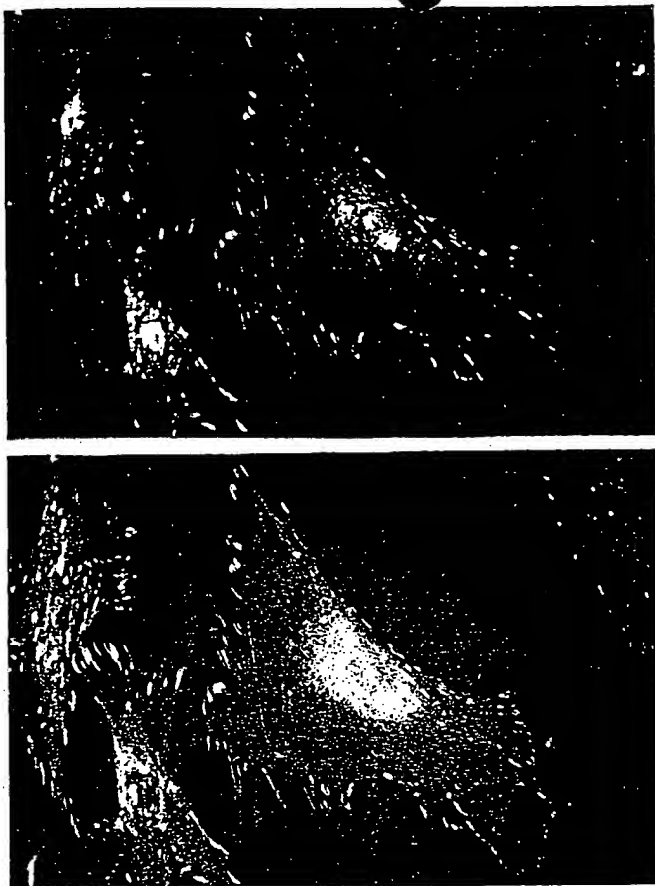


Figure 2. Primary human skin fibroblasts stained with rabbit polyclonal antibodies against type XIII collagen (top), and with monoclonal antibody against vinculin (bottom).

variant of junctional epidermolysis bullosa, usually inherited as an autosomal recessive disorder, that can be caused by mutations in the  $\beta 3$  chain of laminin-5<sup>30</sup> in addition to mutations in  $\alpha 1(\text{XVII})$  collagen. Most of the type XVII collagen mutations described have resulted in premature termination codons in both alleles within the largest triple-helical subdomain. In a Finnish family, the proband was a compound heterozygote, with one allele containing a 5 bp deletion and the other a nonsense mutation.<sup>26</sup> Homozygosity for a mis-sense mutation in type XVII collagen has also been demonstrated in a patient with the localisata variant of junctional epidermolysis bullosa.<sup>31</sup> Detailed and updated information is available through the OMIM database.

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## Other collagens

This is a heterogeneous group of proteins that on a genetic basis do not belong to one of the defined collagen families. They are discussed here as a group only for practical reasons. As the human genome project moves forward it is possible that identification of additional collagen genes will allow classification of the two collagens discussed below as members of their own distinct families.

### Type VI collagen

Type VI collagen is broadly expressed in different tissues as the major component of beaded microfibrils.<sup>1</sup> Each type VI molecule appears in the electron microscope as a 105 nm long triple-helical rod flanked by two globular domains,<sup>2</sup> and contains three different polypeptide subunits  $\alpha 1(\text{VI})$ ,  $\alpha 2(\text{VI})$ , and  $\alpha 3(\text{VI})$ . The three chains have apparent molecular masses of about 140, 130 and 250–350 kDa respectively.<sup>1–3</sup>

These heterotrimeric type VI molecules form disulphide bonded dimers and tetramers. The tetramers associate end-to-end and generate microfibrils, which have a characteristic periodicity of 100 nm.<sup>4–8</sup> The complete primary structures of the  $\alpha 1(\text{VI})$ ,  $\alpha 2(\text{VI})$ , and the  $\alpha 3(\text{VI})$  chains have been determined from amino acid and cDNA sequencing.<sup>9–19</sup> The chains contain a central, relatively short triple-helical domain of 335–336 amino acid residues. All three chains contain a C-terminal non-triple-helical domain composed of two repeats of a 200 residue long segment that is homologous to the A domains of von Willebrand factor. The  $\alpha 3(\text{VI})$  chain contains in addition a proline-rich region showing homology with domains in salivary proteins, a fibronectin type 3 repeat-like domain, and a domain that is similar to a region found in serine protease inhibitors of the Kunitz type.<sup>14,15</sup> In the N-terminal region of the  $\alpha 1(\text{VI})$  and  $\alpha 2(\text{VI})$  chains there is a single 200 residue long von Willebrand factor A homology domain, while the  $\alpha 3(\text{VI})$  chain contains up to nine such repeats in this region. Binding sites for type I collagen have been ascribed to the von Willebrand factor A region,<sup>20</sup> and it is possible that the homologous domains in type VI collagen have collagen binding properties as

well. It is also possible that type VI collagen has a cell adhesion function;<sup>21</sup> several Arg–Gly–Asp sequences are found in the primary sequence of the type VI collagen subunits and experiments with neural crest cells suggest that regions in the N- and C-terminal globular domains play a role in cell adhesion and migration.<sup>22</sup>

Alternative splicing of exons in the 5' region of the  $\alpha 3(\text{VI})$  collagen gene leads to the formation of several transcripts encoding polypeptides with N-terminal globular domains of different size.<sup>18,23–26</sup> Splice variations in the 3' region of the  $\alpha 2(\text{VI})$  gene affecting the structure of the C-terminal globular domain have also been described.<sup>10,17,27</sup>

### Purification and recombinant synthesis

The triple-helical portion of type VI collagen can be obtained by differential precipitation with NaCl from pepsin digests of various tissues in acetic acid or formic acid. Further purification can be accomplished by reprecipitation through dialysis against 0.02 M  $\text{Na}_2\text{HPO}_4$ , followed by ion exchange or molecular sieve chromatography.<sup>11,28</sup> Intact type VI collagen can be purified by ion exchange and molecular sieve chromatography of guanidine or urea extracts of tissues or cell cultures.<sup>1,29</sup> Procedures for isolating intact type VI collagen-containing microfilaments have also been described.<sup>30</sup>

The C-terminal Kunitz-type domain of  $\alpha 3(\text{VI})$  chains has been generated as a recombinant protein and used for structural studies.<sup>31</sup> A large portion of the N-terminal globular domain of  $\alpha 3(\text{VI})$  has also been synthesized as a recombinant protein.<sup>32</sup> All three type VI collagen  $\alpha$  chains have been expressed as recombinant proteins in murine NIH/3T3 cells and shown to assemble into monomers, dimers, and tetramers.<sup>33</sup>

### Antibodies

Several polyclonal and monoclonal antibodies are available against type VI collagen.<sup>1,34–38</sup> They have been used for detecting type VI chains or degradation products by immunoblotting, immunoprecipitation, and immunohis-

tochemistry.<sup>1</sup> Monoclonal antibodies have been used for epitope mapping by rotary shadowing electron microscopy.<sup>1,39</sup> Anti-type VI collagen antibodies are available from several commercial sources. See BioSupplyNet Source Book for suppliers.

## ■ Activities

Type VI collagen molecules assemble into disulphide bonded polymers that form beaded microfibrils.<sup>1,40,41</sup> The microfibrils frequently aggregate further laterally into cross-banded fibres, referred to as Luse bodies, fusiform bodies, or zebra collagen.<sup>1,42,43</sup> Type VI collagen binds to hyaluronan;<sup>44</sup> binding sites for heparin and hyaluronan have been identified within the N-terminal globular domain of  $\alpha 3(\text{VI})$  chains.<sup>32</sup> Type VI collagen also binds to the membrane-associated chondroitin sulphate proteoglycan NG2<sup>45-47</sup> and interacts with the microfibril-associated glycoprotein-1.<sup>48</sup>

## ■ Genes

cDNAs encoding type VI collagen chains in humans, chicken, and mouse have been isolated and sequenced.<sup>19,49,50</sup> The  $\alpha 2(\text{VI})$  gene generates transcripts that are alternatively spliced at the 3' end, giving rise to several mRNA variants.<sup>3</sup> Several variants are also generated by alternative splicing in the 5' region of  $\alpha 3(\text{VI})$  transcripts. The human *COL6A1* and *COL6A2* genes are organized in a head to tail arrangement on chromosome

21q22.3,<sup>51</sup> and both genes have been characterized and compared with the corresponding chicken genes.<sup>52-54</sup>

## ■ Mutant phenotype/disease states

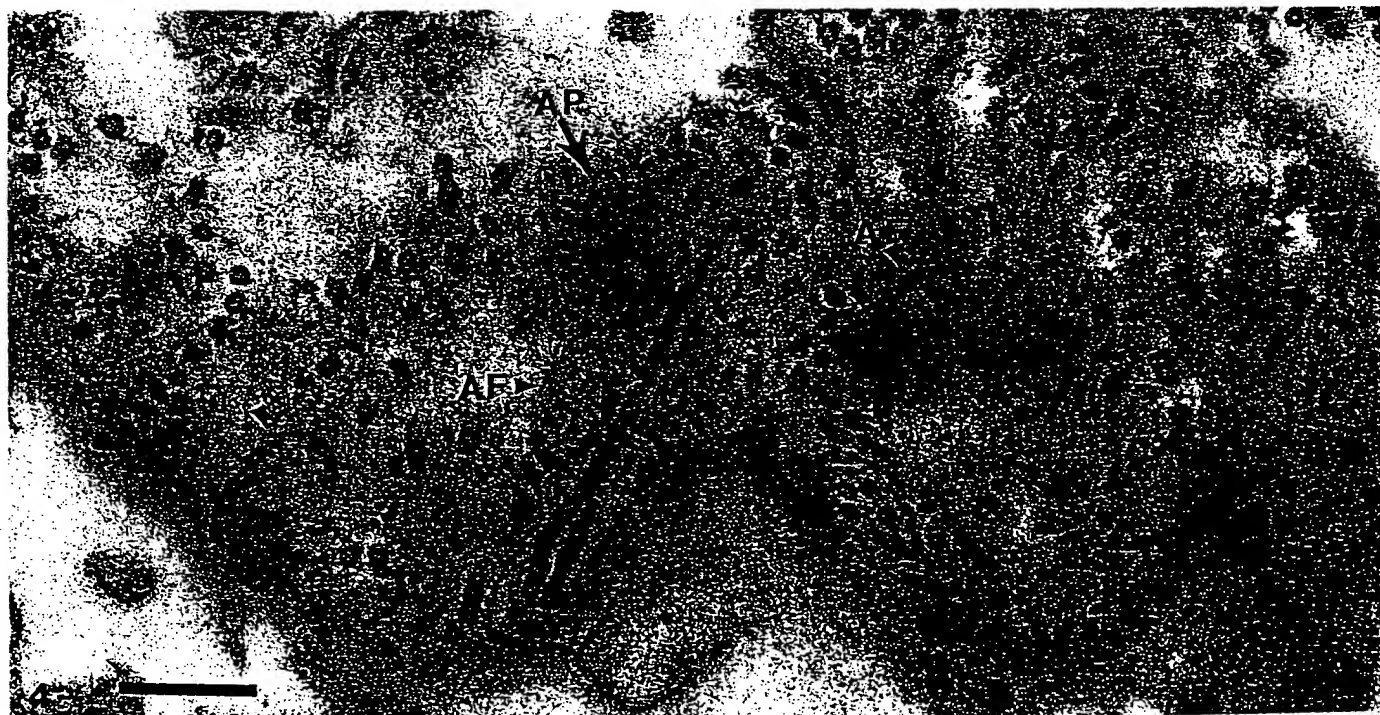
Jobsis *et al.* (1996)<sup>55</sup> demonstrated linkage to the *COL6A1/COL6A2* locus on chromosome 21q22.3 in nine kindreds with the Bethlem form of autosomal dominant myopathy with contractures (OMIM 158810). A mis-sense mutation involving a glycine residue in the triple-helical domain was found in *COL6A1* in one family and in *COL6A2* in two other families.<sup>55</sup> Analysis of a large French Canadian family showed linkage to the *COL6A3* locus on chromosome 2q37.<sup>56</sup> Pan *et al.* have described a missense mutation in *COL6A3*.<sup>93</sup>

## ■ Structure

The crystal structure of the Kunitz-type domain in the C-terminal region of  $\alpha 3(\text{VI})$  chains has been determined at a 1.6 Å resolution,<sup>57</sup> and the solution structure and backbone dynamics of the domain has been analysed by NMR.<sup>58</sup>

## Type VII collagen

Type VII collagen is the major collagenous component of anchoring fibrils associated with the basement mem-



**Figure 1.** Ultrastructural immunolocalization of type VII collagen within the dermal-epidermal junction of neonatal human foreskin with gold-conjugated antibodies. AF, anchoring fibrils; AP, anchoring plaques. (From Keene *et al.* 1987.<sup>62</sup>)

branes under stratified squamous epithelia.<sup>59-61</sup> The fibrils originate from the lamina densa and extend into the upper papillary dermis of skin where they insert into so-called anchoring plaques<sup>62</sup> (Fig. 1). Anchoring fibrils also connect anchoring plaques. Type VII collagen molecules are homotrimers containing a triple-helical domain that is about 50 per cent longer than the triple helix of fibrillar collagens. This domain is flanked by relatively large N- and C-terminal non-triple-helical domains, of molecular masses 150 and 30 kDa, respectively.<sup>60,61</sup> The 30 kDa domain is proteolytically cleaved extracellularly, and the processed molecules form antiparallel dimers, through a C-terminal overlap region. (On the basis of the initial protein studies, the large globular domain was erroneously identified as the C-terminal domain;<sup>63</sup> molecular cloning later showed that the large globular domain was at the N terminus.<sup>64</sup>) Lateral aggregation of such dimers leads to the formation of the centro-symmetrically banded anchoring fibrils.<sup>59</sup>

Keratinocytes are the cells of origin for type VII collagen in skin,<sup>65</sup> and proteolytic processing of the C-terminal globular domain precedes assembly of anchoring fibrils.<sup>66</sup> The N-terminal globular domain has a modular structure,<sup>67</sup> including nine fibronectin type III-like repeats and a von Willebrand factor A-like module.<sup>68</sup> The C-terminal globular domain contains eight cysteines; six of these are contained within a module that is similar to the Kunitz-type module in the C-terminal region of  $\alpha 3(\text{VI})$  collagen chains.<sup>69</sup>

## ■ Purification and recombinant synthesis

The triple-helical domain of type VII collagen can be solubilized by pepsin extraction of human skin or amnion. Purification is by differential salt precipitation with NaCl, followed by ion exchange chromatography and HPLC.<sup>59</sup> The intact, biosynthetic form of type VII collagen has been purified from the media of KB cells (derived from a human oral basal cell carcinoma) and WISH cells (derived from amniotic epithelial cells).<sup>59</sup> Recombinant fusion proteins have been used for epitope mapping and detection of autoantibodies in patient sera.<sup>70-72</sup>

## ■ Activities

Type VII collagen molecules form the anchoring fibrils in skin, chorioamnion, oral mucosa, cornea, and the uterine cervix.<sup>59</sup> Laminin-5,<sup>73</sup> a component of anchoring filaments and a ligand for the integrin  $\alpha 6\beta 4$  within hemidesmosomes, binds to the N-terminal globular domain of type VII collagen.<sup>74</sup> Interactions of the N-terminal domain with fibronectin and type I collagen,<sup>76</sup> and between the type VII collagen triple-helical region and fibronectin,<sup>76</sup> are also likely.

## ■ Antibodies

A number of polyclonal and monoclonal antibodies against type VII collagen are available.<sup>59-79</sup> Auto-

antibodies from patients with acquired epidermolysis bullosa have been shown to react with specific epitopes in  $\alpha 1(\text{VII})$  collagen chains.<sup>70-72,80</sup>

## ■ Genes

Screening of a cDNA expression library with autoantibodies against type VII collagen from a patient with acquired epidermolysis bullosa resulted in the first isolation of human  $\alpha 1(\text{VII})$  collagen cDNA.<sup>81</sup> This led to the isolation of cDNAs covering the entire mRNA,<sup>68,69</sup> and characterization of the entire human *COL7A1* gene.<sup>82</sup> The mouse cDNA and *Col7a1* gene has also been characterized.<sup>83,84</sup>

## ■ Mutant phenotype/disease states

The epidermolysis bullosa group of inherited blistering diseases in humans is classified into simplex, junctional, and dystrophic forms. The simplex forms are caused by mutations in keratins 5 and 14,<sup>85-87</sup> the junctional forms are caused by mutations in laminin-5,<sup>88</sup> and the dystrophic forms are the consequences of mutations in type VII collagen.<sup>88</sup> The mutations in *COL7A1* range from premature termination codons resulting in severe, mutilating recessive dystrophic epidermolysis bullosa of the Hallopeau-Siemens type (OMIM 226600)<sup>89,90</sup> to glycine substitutions in the triple-helical region of  $\alpha 1(\text{VII})$  collagen resulting in clinically less severe, dominant or recessive, dystrophic epidermolysis bullosa.<sup>91</sup> A clinical variant of dominant dystrophic epidermolysis bullosa called the Bart syndrome (OMIM 132000) is caused by a glycine-substitution mutation in  $\alpha 1(\text{VII})$  collagen.<sup>92</sup> An updated listing of all mutations in type VII collagen can be found in the OMIM database (OMIM 120120 collagen).

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## Decorin

Decorin (DCN) is a small proteoglycan composed of a ~38 kDa core protein usually modified with a single chondroitin sulphate (bone) or dermatan sulphate (most soft tissues) glycosaminoglycan chain and two or three *N*-linked oligosaccharides. DCN is virtually ubiquitous in the matrices of various connective tissues, being found bound to or 'decorating' the collagen fibrils. The protein portion is composed of 10 tandem repeats of ~25 amino acids characteristically rich in ordered leucines with the repeats being flanked by two cysteine disulphide loops. These tandem repeats are found a wide variety of closely related small proteoglycans including: biglycan (BGN), fibromodulin, lumican, epiphykan, keratocan, and PG-Lb. The most commonly cited functions of DCN are its roles in collagen fibril assembly (and stabilization) as well as its ability to bind to TGF- $\beta$ .

### ■ Synonymous names

Decorin has several synonymous names, most reflecting its relative position on SDS-PAGE or time of elution from various purification columns. The names include PG40, PG-2, PG-II, PG-S2, CS-PGII, and DS-PGII.

### ■ Protein properties

Decorin is a member of a growing family of small proteoglycans whose unifying characteristics are two highly conserved cysteine loops flanking 5 to 10 tandem repeats. Each repeat is nominally ~25 amino acids in length and is based on the pattern LxxLxLxxNxLx<sub>(12-14)</sub>. For DCN there are 10 repeats and the single glycosaminoglycan (GAG) chain is chondroitin sulphate in bone matrix and dermatan sulphate in most soft tissues. Other members of this family include biglycan, fibromodulin, lumican, epiphykan, keratocan, and PG-Lb (known as DSPG3 in human) (for a review see ref. 1). The DCN sequences from a number of species have been reported, including human,<sup>2</sup> cow,<sup>3</sup> mouse,<sup>4</sup> rat,<sup>5</sup> rabbit,<sup>6</sup> and chicken.<sup>7</sup> Curiously, the chicken form can have two GAG chains and these chains appear to be attached to a GlySer sites rather than the apparently universal mammalian Ser-Gly.<sup>7</sup> Using human DCN as the model, decorin has 359 amino acids (~39 700 Da) including 17 in the leader sequence and 14 more in the amino terminus that are often removed and are therefore considered to be a propeptide region.<sup>2</sup> The 'mature' core protein (lacking the propeptide), made by removing the disaccharide

## $\alpha 1$ (XVIII), a collagen chain with frequent interruptions in the collagenous sequence, a distinct tissue distribution, and homology with type XV collagen

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EXHIBIT

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**ABSTRACT** We report on the isolation of mouse cDNA clones which encode a collagenous sequence designated here as the  $\alpha 1$  chain of type XVIII collagen. The overlapping clones cover 2.8 kilobases and encode an open reading frame of 928 amino acid residues comprising a putative signal peptide of 25 residues, an amino-terminal noncollagenous domain of 301 residues, and a primarily collagenous stretch of 602 residues. The clones do not cover the carboxyl-terminal end of the polypeptide, since the translation stop codon is absent. Characteristic of the deduced polypeptide is the possession of eight noncollagenous interruptions varying in length from 10 to 24 residues in the collagenous amino acid sequence. Other features include the presence of several putative sites for both N-linked glycosylation and O-linked glycosaminoglycan attachment and homology of the amino-terminal noncollagenous domain with thrombospondin. It is of particular interest that five of the eight collagenous sequences of type XVIII show homology to the previously reported type XV collagen, suggesting that the two form a distinct subgroup among the diverse family of collagens. Northern blot hybridization analysis revealed a striking tissue distribution for type XVIII collagen mRNAs, as the clones hybridized strongly with mRNAs of 4.3 and 5.3 kilobases that were present only in lung and liver of the eight mouse tissues studied.

The collagens comprise a large family of heterotrimeric or homotrimeric triple-helical proteins that constitute the major structural components of the extracellular matrix. Several other proteins are known to contain short triple-helical collagen domains but are not classified as collagens, as they do not participate in assembly of the extracellular matrix (1-3). The vertebrate collagens can be divided into two groups, fibrillar and nonfibrillar, on the grounds of their primary structure and supramolecular assemblies (1, 2). All collagen molecules contain a central collagen domain consisting of repeating Gly-Xaa-Yaa triplets and noncollagenous domains at their termini. The fibrillar group comprises the classical collagens, types I-III, and types V and XI. These molecules contain collagenous domains of about 1000 aa, highly conserved carboxyl-terminal noncollagenous domains of about 250 aa, and variable amino-terminal noncollagenous domains of 50-520 aa. The fibrillar collagens participate in highly ordered quarter-staggered fibrils that provide tensile strength for the tissues.

The nonfibrillar group comprises collagen types IV, VI-X, and XII-XVII (1-3). These molecules display great heterogeneity in structure, tissue location, macromolecular organization, and function. On common feature is that they all have one or more interruptions in the collagenous sequence. Their collagenous sequences vary in length from about 330 to 1400 aa, the shortest being found in type VI collagen mole-

cules and the longest in type VII. Their carboxyl-terminal and amino-terminal noncollagenous domains also are highly variable in both sequence and length, the latter varying in both domains from <20 aa to several hundred amino acids. One subgroup among the nonfibrillar collagens is formed by the fibril-associated collagens with interrupted triple helices (FACIT): types IX, XII, and XIV (2). These collagens share sequence homology and do not appear to form polymers alone but are associated with fibrils composed of fibrillar collagens. Another subgroup is formed by the structurally homologous types VIII and X, which are thought to form sheets in the extracellular matrix (2). The recently described types XV (4), XVI (5), and XVII (6) differ from the other nonfibrillar collagens in being characterized by numerous interruptions in their triple-helical regions. Type XVI collagen shares some structural features with the FACIT collagens, as also does another recently characterized form called Y-collagen (7, 8). Type XVII (6), a hemidesmosomal protein also known as the 180-kDa bullous pemphigoid antigen, is unique among the collagens in that it is thought to be a transmembrane protein.

Collagen types XIII and XV and the  $\alpha 5$  chain of type IV collagen were identified in our laboratory by screening of cDNA libraries under low stringency with probes encoding collagenous sequences (4, 9, 10). Recently we screened a mouse cDNA library to obtain clones coding for the mouse counterpart to the previously characterized human type XIII collagen (9). One of the positive clones was found to encode a collagenous protein not described before.† We present here a partial characterization of this polypeptide, which is characterized by multiple interruptions in the triple helix, and suggest that it should be designated the  $\alpha 1$  chain of type XVIII collagen. Our findings indicate that type XVIII collagen has an unusual tissue location. Furthermore, type XVIII was found to be homologous with type XV, and the two thus form a subgroup among the collagens.

## MATERIALS AND METHODS

**Isolation of cDNA Clones and DNA Sequencing.** A 500-bp clone, G2, encoding the  $\alpha 1$  chain of murine type XIII collagen (unpublished results) was used as a probe to screen a mouse embryo Agt11 cDNA library (ML 1027a, Clontech) under stringent conditions (11). The final wash for the filters was at 50°C in 0.5× standard saline citrate (SSC)/0.1% NaDodSO<sub>4</sub>. The recombinant phage ME-1 was isolated and the insert DNA was subcloned to the EcoRI site of pBluescript SK (Stratagene). The nucleotide sequence was determined for

Abbreviation: FACIT, fibril-associated collagen(s) with interrupted triple helices.

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†The sequences reported in this paper have been deposited in the GenBank database (accession no. L16898).

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both strands of the cDNA by the dideoxynucleotide method (12) using the enzyme Sequenase (United States Biochemical) and vector or insert-specific primers. The same library was screened with the ME-1 cDNA under stringent conditions as above but the final wash was at 65°C in 0.5× SSC. The positive recombinant phages were isolated and characterized as above.

**Northern Blot Analysis.** A mouse multi-tissue Northern blot (Clontech) containing 2 µg of poly(A)<sup>+</sup> RNA per sample isolated from various adult mouse tissues was hybridized under stringent conditions with the <sup>32</sup>P-labeled probe SXT-5. Hybridization was carried out as suggested in the manufacturer's protocol except that the final wash at 65°C was in 0.2× SSC instead of 0.1× SSC. The intactness of the RNA samples on the blot was checked with the β-actin probe provided with it. The band intensities were scanned with a BioImage densitometer (Millipore).

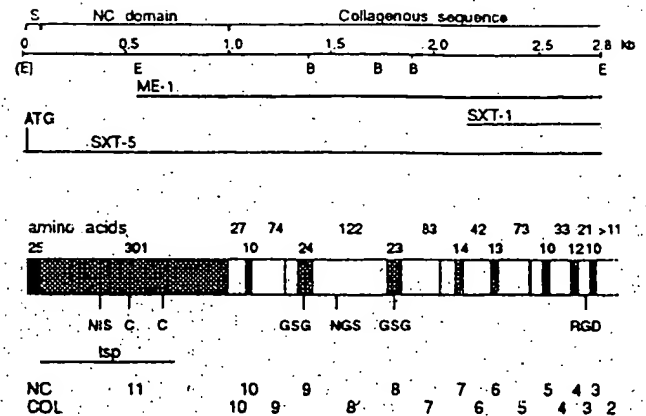
**Sequence Analysis.** Nucleotide and amino acid homology comparisons were carried out against the GenBank, EMBL, PIR, and Swiss-Prot databases at the National Center for Biotechnology Information with the BLAST network service (13). The search for functional patterns of amino acid sequences was carried out with the PROSITE database (14).

## RESULTS AND DISCUSSION

**Isolation of Mouse cDNA Clones Encoding the α1(XVIII) Collagen Chain.** A 500-bp cDNA, G2, that encodes the α1 chain of mouse type XIII collagen (unpublished results) was used as a probe to screen an 11.5-day mouse embryo cDNA library. Five positive signals were identified among ~900,000 clones. One of these, ME-1, contained a 2.3-kb cDNA insert that coded for a collagenous polypeptide. Rescreening of about 600,000 recombinants of the same library with ME-1 resulted in the identification of 2 additional clones, SXT-1 and SXT-5, with inserts of 0.6 kb and 2.8 kb, respectively. Together these 3 clones cover 2.8 kb of the corresponding mRNA sequence (Fig. 1). The nucleotide and amino acid sequences derived from them were not compatible with any of the previously characterized collagens I–XVII or any other reported collagenous sequence (1–10). It is thus proposed that the polypeptide encoded by the clones should be designated the α1 chain of type XVIII collagen.

**Partial Nucleotide and Amino Acid Sequences of the Mouse and Human α1(XVIII) Collagen Chains.** The mouse clones encode an open reading frame of 928 aa preceded by 20 nt of 5' untranslated sequence (Fig. 2). The other reading frames contain multiple stop codons. The presumed translation initiation codon is encoded by nt 21–23. Sequences surrounding the codon for methionine match well with the best-conserved nucleotides (underlined) of the proposed consensus sequence for initiation of translation, GCC(R)CCAUGG (15). The amino-terminal end of the predicted polypeptide contains a hydrophobic sequence that clearly fulfills the criteria for a signal peptide, and on comparison with other proteins this sequence was found to be highly homologous with the signal peptide of decorin, the identity being 80% among the 10 residues preceding the proposed cleavage site for human decorin (data not shown; for decorin sequence, see ref. 16). Thus, comparisons with other proteins and prediction of the signal-peptide cleavage site by the method of von Heijne (17) led to the suggestion that the α1(XVIII) collagen chain has a signal peptide of 25 aa. Positions –3 and –1 are occupied by serine and alanine, residues frequently found in these positions (17). The presence of the signal peptide suggests that the polypeptide is secreted into the extracellular matrix.

The putative signal peptide is followed by a 301-aa noncollagenous domain that contains the only cysteine residues of the portion of the polypeptide encoded by the clones de-



**FIG. 1.** cDNA clones encoding part of the mouse α1 chain of type XVIII collagen and schematic structure of the deduced polypeptide. (Upper) The overlapping cDNA clones ME-1, SXT-1, and SXT-5 and the locations of the EcoRI (E) and BamHI (B) restriction sites. The EcoRI site shown in parentheses represents a linker site introduced during cloning. (Lower) cDNA-derived polypeptide structure. The numbering of the noncollagenous (NC) and collagenous (COL) domains is shown below the polypeptide, and the lengths of these domains in amino acids are given above the polypeptide. The numbering of the domains begins from the carboxyl end of the polypeptide, based on carboxyl-terminal sequence characterized by Oh *et al.* (18). The dashed lines indicate that the clones do not cover the carboxyl-terminal end of the polypeptide. Thus, the COL2 domain is expected to be >11 aa. Dark box, signal peptide; ATG, putative translation initiation codon; stippled boxes, noncollagenous sequences; open boxes, collagenous sequences; NIS and NGS, potential N-linked glycosylation sites; GSG, potential O-linked glycosylation sites; C, cysteine; RGD, potential cell attachment site; tsp, thrombospondin homology area.

scribed here (Fig. 2). The rest of the sequence consists of a 602-aa primarily collagenous sequence (Fig. 2). The clones do not fully cover the carboxyl-terminal end of the predicted polypeptide, however, since the stop codon is lacking. A notable feature of the collagenous sequence is that it contains eight interruptions. The eight collagenous domains interspersed by the interruptions range in size from 21 to 122 aa, and five of the seven noncollagenous domains vary in size from 10 to 14 aa, while two are longer ones, of 23 and 24 aa. Furthermore, the four longest collagenous domains contain a total of five short imperfections that are due to the lack of one residue of the collagenous Gly-Xaa-Yaa triplet. The collagenous sequences are rich in proline, as this amino acid residue represents 27% of all the residues in the Gly-Xaa-Yaa triplets. Fifty-eight percent of the prolines are in the Yaa position and, thus, are subject to 4-hydroxylation (3). The polypeptide structure is presented schematically in Fig. 1, with the noncollagenous and collagenous domains numbered from the carboxyl-terminal end.

Oh *et al.* (18) have independently isolated cDNAs that also code for the polypeptide described here. The 5' sequences of their clones differ from the first 99 nt of our clones, which may indicate that the α1(XVIII) gene has alternative promoters or that its transcripts are subject to alternative splicing. As the clones by Oh *et al.* (18) cover the carboxyl-terminal end of the polypeptide, it can be estimated that our clones cover only part of the penultimate collagenous domain and lack sequences corresponding to the last collagenous domains and the carboxyl-terminal noncollagenous domain.

**Types XVIII and XV Form a Subclass Within the Collagen Family.** Since the total number of the residues in the interruptions is 113, 19% of the residues in the portion of the α1(XVIII) collagenous sequence described here are not located in the Gly-Xaa-Yaa repeats. The fact that it contains frequent interruptions in the collagenous sequence means

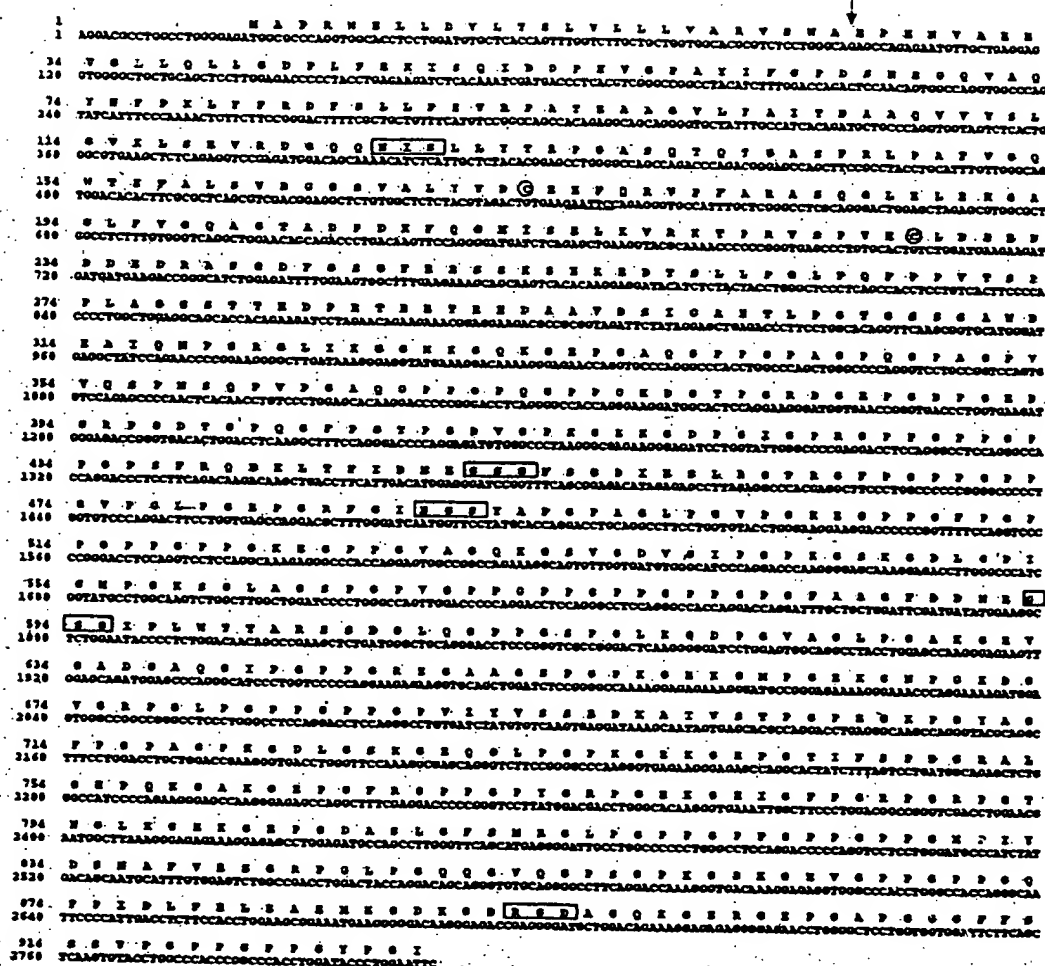


FIG. 2. Nucleotide and deduced amino acid sequences of cDNA clones for mouse  $\alpha 1$  chain of type XVIII collagen. Noncollagenous sequences are shaded. The N(I/G)S and GSG sequences which may serve as sites for N-linked and O-linked glycosylation, respectively, are boxed, as is a potential cell attachment site, RGD. Arrow indicates the putative signal peptide cleavage site. Cysteine residues are circled and *EcoRI* sites are underlined.

that type XVIII collagen resembles three other collagen chains:  $\alpha 1(XV)$ ,  $\alpha 1(XVI)$ , and  $\alpha 1(XVII)$ . The  $\alpha 1(XV)$  chain has a 577-aa collagenous domain with 8 interruptions containing 33% of the collagenous-domain residues (4),  $\alpha 1(XVI)$  a 1244-aa collagenous domain with 9 interruptions containing 15% of the collagenous residues (5), and  $\alpha 1(XVII)$  an 846-aa collagenous domain with 12 interruptions hosting 36% of the residues (6). The human  $\alpha 1(XV)$  chain has been reported to consist of nine collagenous domains, termed here COL9-COL1 (numbered from carboxyl terminus to amino terminus), with sizes of 18, 114, 35, 45, 71, 30, 18, 55, and 15 aa.

respectively (4). The four extreme carboxyl-terminal collagenous domains fully covered by the mouse  $\alpha 1(\text{XVIII})$  clones are 42, 73, 33, and 21 aa [COL6-COL3 in Fig. 1, numbered from the carboxyl-terminal end of the polypeptide as suggested by Oh *et al.* (18)], being thus similar in size to the four underlined collagenous domains of the  $\alpha 1(\text{XV})$  chain. Closer comparison indicates that these four collagenous domains of the  $\alpha 1(\text{XVIII})$  and  $\alpha 1(\text{XV})$  chains are homologous in their amino acid sequences (Fig. 3), this homology being most notable between the 71-residue COL5 domain of  $\alpha 1(\text{XV})$  and the 73-residue COL5 of  $\alpha 1(\text{XVIII})$ , with 59% identity. The

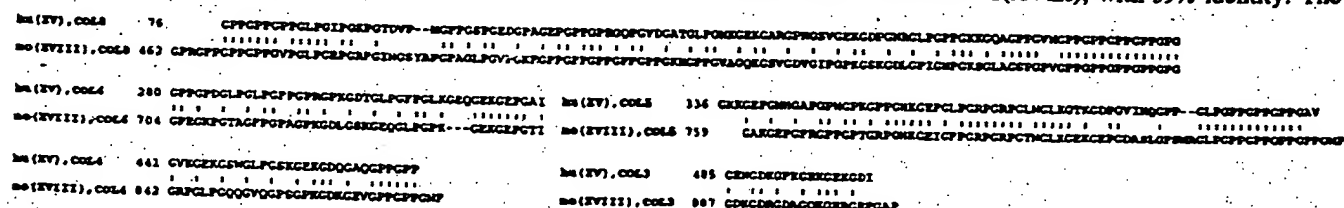


FIG. 3. Comparison of homologous collagenous sequences between mouse  $\alpha 1(\text{XVIII})$  and human  $\alpha 1(\text{XV})$  chains. The number of the collagenous (COL) domain aligned is indicated for both chains, the numbering beginning from the carboxyl termini of the chains. The gaps indicated by dashes were introduced for maximal alignment of the chains. The numbers of amino acid residues are given by counting from the initiation methionine for the  $\alpha 1(\text{XVIII})$  chain (see Fig. 2) and from the first amino acid residue reported for the partially characterized  $\alpha 1(\text{XV})$  chain (4). The aligned domains are similar in size and, to varying extents, homologous in amino acid sequence. Two of the aligned pairs also contain similarly located short imperfections (indicated in bold type) in the repeating Gly-Xaa-Yaa sequences.





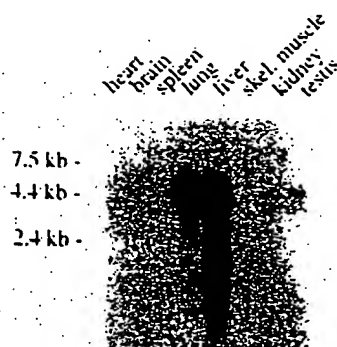


FIG. 5. Northern blot analysis of  $\alpha 1(\text{XVIII})$  collagen mRNAs in mouse tissues. Each lane contained 2  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA from the adult mouse tissues indicated. The blot was hybridized to a mouse  $\alpha 1(\text{XVIII})$  collagen clone. Autoradiography time was 18 hr.

tively spliced forms of the  $\alpha 1(\text{XVIII})$  mRNA. Another explanation, however, may be that they are transcripts of a different gene. The same blot was also hybridized with a  $\beta$ -actin probe to confirm the intactness of the mRNAs isolated from each tissue. Strong hybridization signals were obtained from each tissue, with no sign of degradation of the RNAs (data not shown); thus excluding the possibility that the lack of  $\alpha 1(\text{XVIII})$  transcripts in most tissues could be an artifact.

**Conclusions.** The mouse clones described here code for a unique polypeptide, designated as the  $\alpha 1$  chain of type XVIII collagen. Altogether, 928 aa were determined, including a signal peptide of 25 aa, an amino-terminal noncollagenous domain of 301 aa, and a 602-aa stretch of a collagenous region. Type XVIII collagen was found to resemble type XV collagen (4) in containing multiple interruptions and imperfections in the collagenous sequences and in having several sequences that may serve as sites for N- and O-linked glycosylation. Several of the variable-length collagenous domains of the two types were found to be similar in both size and sequence, leading to the suggestion that collagen types XVIII and XV form a subclass within the large family of collagenous proteins.

The amino-terminal noncollagenous domain of type XVIII collagen contained an  $\approx 200$ -aa sequence that was homologous to thrombospondin. It has been reported that collagen types V, IX, XI, XII, and XIV contain this sequence module (22, 24), and we found it to be the only homology between type XVIII and the other collagens except for type XV. Thus, molecules belonging to different subclasses of collagens—i.e., the fibrillar and FACIT collagens, and also some other collagens,—share this sequence module, although its functional significance in collagens is not known. Of interest is that the two cysteine residues that are conserved within this sequence in all collagens are known to form a disulfide bond in a proline- and arginine-rich protein, PARP (25). It thus seems likely that the only two cysteines found in the amino-terminal noncollagenous domain of the  $\alpha 1(\text{XVIII})$  chain also take part in disulfide bond formation.

Type XVIII collagen mRNAs had a striking tissue distribution, as demonstrated by the clear Northern signal in liver and lung RNA but not in brain, heart, kidney, skeletal muscle, spleen, or testis RNA. Further research will be required, however, to obtain a complete picture of the pattern

of expression of this collagen. The present finding of marked amounts of mRNAs only in liver and lung among the eight mouse tissues studied already justifies the suggestion that type XVIII collagen mRNAs have a distinct tissue distribution that is not similar to that of any of the previously described collagens.

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